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16. Abstract <p>The Annual Report presents the fiscal year (FY) 1989 research activities and accomplishments for the Biocatalysis Project of the U.S. Department of Energy, Energy Conversion and Utilization Technologies (ECUT) Division. The ECUT Biocatalysis Project is managed by the Jet Propulsion Laboratory, California Institute of Technology, under the direction of the DOE ECUT Biocatalysis Program Manager. The Biocatalysis Project is a mission-oriented, applied research and exploratory development activity directed toward resolution of the major generic technical barriers that impede the development of biologically catalyzed commercial chemical production. The approach toward achieving project objectives involves an integrated participation of Universities, Industrial Companies and Government Research Laboratories.</p> <p>The Project's technical activities were organized into three work elements: (1) molecular modeling and applied genetics, (2) bioprocess engineering, and (3) bioprocess design and assessment.</p>			
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ENERGY CONVERSION AND UTILIZATION TECHNOLOGIES PROGRAM

Biocatalysis Project Annual Report FY 1989

May 1990

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The Biocatalysis Project focuses on resolving the major technical barriers that impede the potential use of biologically-facilitated continuous chemical production processes.

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ABSTRACT

The Annual Report presents the fiscal year (FY) 1989 research activities and accomplishments, for the Biocatalysis Project of the U.S. Department of Energy, Energy Conversion and Utilization Technologies (ECUT) Division. The ECUT Biocatalysis Project is managed by the Jet Propulsion Laboratory, California Institute of Technology, under the direction of the DOE ECUT Biocatalysis Program Manager. The Biocatalysis Project is a mission-oriented, applied research and exploratory development activity directed toward resolution of the major generic technical barriers that impede the development of biologically catalyzed commercial chemical production. The approach toward achieving project objectives involves an integrated participation of Universities, Industrial Companies and Government Research Laboratories.

The Project's technical activities were organized into three work elements:

The Molecular Modeling and Applied Genetics work element includes research on modeling of biological systems; developing rigorous methods for the prediction of three-dimensional (tertiary) protein structure from the amino acid sequence (primary structure) for designing new biocatalysts; defining kinetic models of biocatalyst reactivity; and developing genetically engineered solutions to the generic technical barriers that preclude widespread application of biocatalysis.

The Bioprocess Engineering work element supports efforts in novel bioreactor concepts that are likely to lead to substantially higher levels of reactor productivity, product yields and lower separation energetics. Results of work within this work element will be used to establish the technical feasibility of critical bioprocess monitoring and control subsystems.

The Bioprocess Design and Assessment work element attempts to develop procedures (via user-friendly computer software) for assessing the energy-economics of biocatalyzed chemical production processes, and initiation of technology transfer for advanced bioprocesses.

FOREWORD

For further information about the ECUT Biocatalysis Project, contact:

Dr. Leonard Keay,
Biocatalysis Program Manager
Energy Conversion and Utilization
Technologies (ECUT) Program
Conservation and Renewable Energy
Department of Energy CE-12
Forrestal Bldg. 5E-052
1000 Independence Avenue, S.W.
Washington, D.C. 20585
(FTS) 896-1493 (202) 896-1493

Dr. Gene Petersen,
ECUT Biocatalysis Project
Field Manager
Jet Propulsion Laboratory
California Institute of
Technology
M/S 89-2
4800 Oak Grove Drive
Pasadena, CA 91109
(818) 354-7019
(FTS) 792-7019

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SECTION I

EXECUTIVE SUMMARY

The Biocatalysis Project sponsored by the Energy Conversion and Utilization Technologies (ECUT) Division of the U.S. Department of Energy (DOE) is an applied research effort focused on providing the enabling technology base for new bioprocess applications which have substantial energy implications. The primary mission of DOE-ECUT is to provide, for the nation, a bridge between basic research and industrial applications for several technologies (e.g., high temperature materials, tribiology, combustion and biocatalysis) which have the potential for making positive energy contributions. Furthermore, the ECUT mission hopes to strengthen the future U.S. competitive position by conducting research directed towards exploiting appropriate advances in basic research in which the U.S. has demonstrated leadership.

As the national lead center, the Jet Propulsion Laboratory of the California Institute of Technology serves as the technical field manager for the Biocatalysis Project. The primary objective of the Project is to resolve the critical technical constraints (e.g., poor productivity, high separation energetics, presence of aqueous medium, lack of design tools) that impede the utilization of biocatalysis for the production of chemicals and materials. To achieve this objective, the Project supports generic applied research as well as development of predictive theoretical models (that are experimentally verifiable) which address the technological barriers to the commercial utilization of biochemical catalysis.

The ECUT Biocatalysis Project consists of three major work elements, each addressing a key technical component of the required enabling technology base. The selection as well as the relationship between each of these three work elements has been defined by their scale of action. The Molecular Modeling and Applied Genetics work element focuses on defining optimal microscale parameters for biocatalysis and pursuing practical applications of basic molecular biology research findings. Computer graphic models have been advanced which predict the dynamic behavior and intramolecular conformational changes of enzymes (biocatalysts), including effects of temperature and biocatalytic inhibitions. Advances in Applied Genetics have focused on the issue of biological information (plasmid) stability. The Bioprocess Engineering work element emphasizes defining the basic engineering relationships between cellular scale events and macro-level parameters. Laboratory tests using advanced fluidized bed bioreactors, novel multi-membrane bioreactors and chemical transformation in organic solvents have substantially increased rates and the extent of chemical synthesis by solving the chronic and ubiquitous problem of

product feedback inhibition. The Process Design and Analysis work element focuses on developing user friendly computer programs which assess the energetics and economics of biocatalytic chemical production processes. Modular computer programs have been developed which provide capabilities for rapid assessment of energy expenditures and costs for unit operations within biocatalytic processes.

SECTION II

PROJECT DESCRIPTION

A. THE ECUT MISSION

The Energy Conversion and Utilization Technologies (ECUT) Program seeks to:

- (1) Monitor advances in basic scientific research and evaluate emerging technologies for applicability to energy conservation.
- (2) Conduct exploratory development and establish feasibility of innovative, or revolutionary, conservation concepts.
- (3) Effect technology transfer to DOE end-use conservation programs and/or to private industry.
- (4) Identify energy conservation research needs and develop programmatic direction in basic research.

In fulfilling these aims ECUT acts to transmit the power of basic research to the wheels of industry (Figure 2-1). There are many ways to achieve these aims and since its establishment ECUT has chosen to support long-term, high-risk generic research and exploratory development for generation of more energy-efficient materials and processes. The Biocatalysis Project is one of five projects that make up the ECUT Program (Figure 2-2).

B. HISTORICAL BACKGROUND

The Biocatalysis Project was established in 1980 as the Chemical Processes Project with the Jet Propulsion Laboratory as the lead center. At that time the Project had two work elements: (1) Catalysis (chemical catalysis and biocatalysis) and (2) Separation. Within these work elements several small contracts were completed that evaluated the energy efficiencies and potential applications of relatively new separation processes, such as supercritical extraction, membrane separation, and chromatography. Research was also started at the California Institute of Technology (Caltech) on chemical catalytic behavior models and kinetics of expression in recombinant-DNA microorganisms.

In 1982, the ECUT Program was reorganized and the Chemical Processes Project was renamed as the Biocatalysis Project. As the name suggests, the Project emphasis was also changed to stress biocatalysis as the primary research focus. Its work elements were modified to include: (1) Molecular Modeling and

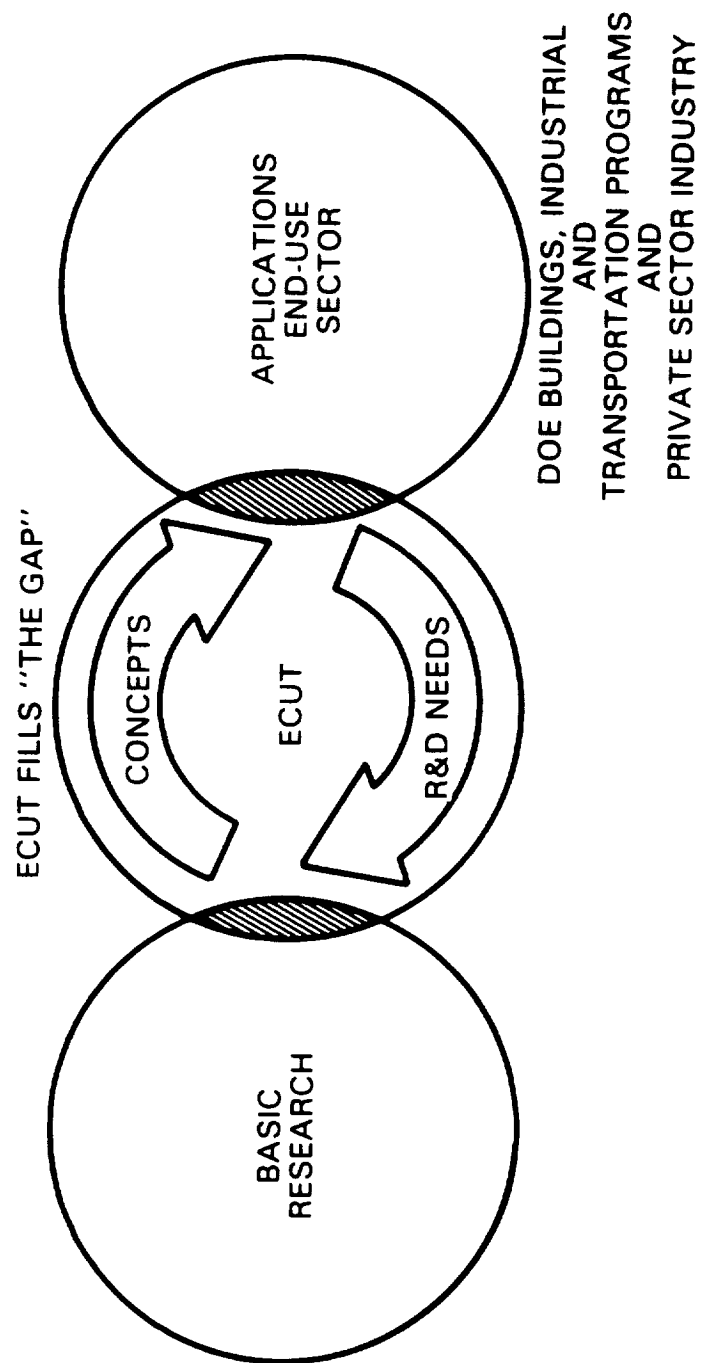


Figure 2-1. ECUT Role in Energy Conservation Research and Development

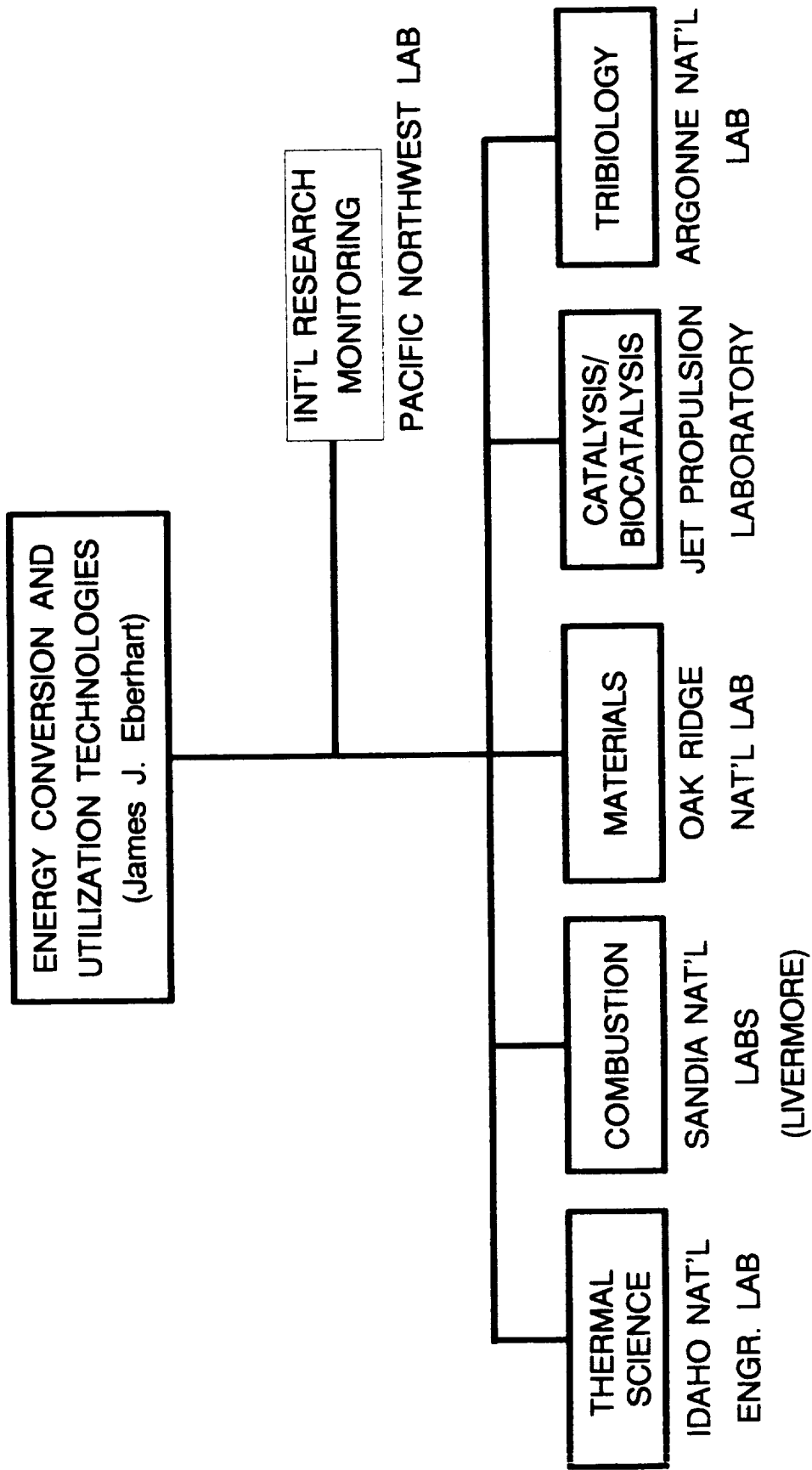


Figure 2-2. ECUT Program: Projects and Lead Center

Applied Genetics, (2) Bioprocess Engineering, and (3) Process Design and Analysis. Each of these elements addresses a key technical component necessary for the development of more advanced and efficient bioprocesses. These three work elements detail a systematic progression of understanding starting with the basic understanding of biocatalytic mechanisms at the molecular level, to micro-level effects on process parameters in reactors (including effects of integrated separation processes), and finally to the development and assessment of new process concepts for technology transfer to the industrial sector. With this information the production of large-volume, low-cost, energy-intensive chemicals from renewable resources may be a practical alternative in the future.

The selection as well as the relationships between each of the work elements has been defined by their scale of action. Research activities are conducted at various scales of action or specific size dimensions. Hence Work Element (1), Molecular Modeling and Applied Genetics, includes research activities at a molecular and cellular level, i.e., a scale of 1 μm and smaller. To successfully exploit the findings at the molecular and cellular level, Work Element (2), Bioprocess Engineering, conducts research in the area of engineering kinetics and control as well as novel concepts in reactor design. The scale in this work element is generally at the 1 meter dimension, or at the reactor level. Finally, Work Element (3), Process Design and Analysis, focuses on activities that operate at the entire process level (hundreds of meters or more). A central tenet subscribed to by this Project is that in the acquisition of an appropriate enabling technology, the Project will integrate crucial research findings within the various work elements and transfer these findings to the private sector.

C. RELEVANCE

Two main conclusions can be stated regarding the competitive status and future potential of the United States Petrochemical Industry:

- (1) The United States must take advantage of its competitive strengths if it is to compete effectively in the future. This includes the application of its world renowned capabilities in basic science to commercial applications. Biotechnology is an example of scientific research whose application has tremendous potential for commercial application.
- (2) Although biotechnology is being applied towards the production of specialty chemicals by the chemical industry, its application to the far larger commodity chemical market is currently considered too long term and risky to attract research and development dollars.

This approach is sensible from the perspective of each individual firm, but it is unclear whether the specialty chemical markets can sustain all of the competitors who are restructuring their operations towards specialty chemical production. A key government role in the future competitiveness of the industry should be the support of generic research which can be applied to the development of economical commercial processes.

The application of biocatalytic processes to the production of large scale chemicals would capitalize on the three major areas in which the United States currently exerts leadership and whose successful exploitation would contribute to its global competitive position in the future. First, the United States is considered a world leader in basic research advances and our lead in molecular biology and biochemistry is unquestioned. Commercial utilization of that lead could increase the competitive advantage of our domestic chemical industries. Second, we can exert our leadership further by using the tools of macromolecular modeling and energy optimization to design entirely novel biocatalysts and to test such catalysts through computer simulation. Such a design strategy would be useless without a means for inexpensive synthesis; however, the techniques of site-directed mutagenesis may provide the methodology to synthesize and test experimentally these design principles. The use of this design strategy would enhance our productivity in the future by significantly and intelligently reducing the time spent on experimental and empirical procedures in the laboratory. Third, the United States is one of the most efficient and successful producers of agricultural products in the world. Because biomass is often the feedstock for the biocatalyst production of organic chemicals, we have a clear competitive advantage in biomass production within our domestic agricultural sector.

D. GOAL AND OBJECTIVE

- (1) Goal. The goal of the ECUT/Biocatalysis Project is to exploit the United States' competitive advantage in biotechnology by facilitating the production of chemicals efficiently via biocatalytic processes. The project supports high risk applied generic research and development aimed at advancing an enabling technology base that will allow the rational development and scale-up of large biocatalyst chemical production processes. The Biocatalysis Project does this by conducting exploratory development on, and by establishing the feasibility of, novel, innovative or revolutionary basic research advances in biotechnology.
- (2) Objective. To meet this goal, the Biocatalysis

Project's approach is to focus on developing predictive models and supporting novel bioprocessing concepts which can be utilized by commercial producers for large scale chemical production. In addition, the strategy of the Biocatalysis Project is to reduce production costs by increasing product yields, by increasing reactor productivity, and by decreasing energy requirements for production. These concerns are generic in a very large number of biocatalytic processes.

E. PROJECT STRUCTURE AND ORGANIZATION

The activities of the Biocatalysis Project have been incorporated as three major work elements and a Management Support Function. Each work element addresses a key technical component for the development of the enabling technology base to produce large volumes of low priced, energy intensive bulk chemicals. The three major work elements and their supporting tasks are:

- (1) The Molecular Modeling and Applied Genetics work element focuses on defining optimal microscale parameters for biocatalysts and developing practical applications of basic molecular biology research findings. The primary roles of this work element are: to model biological systems and develop rigorous methods for the prediction of three dimensional structure from the amino acid sequence of proteins for design of new biocatalysts, to provide a data base for defining kinetic models of biocatalyst reactivity, and to develop genetically engineered solutions to the generic technical barriers that preclude widespread applications of biocatalysis. FY 1989 tasks included:
 - (a) Theory of Biocatalysis.
 - (b) Metabolic Engineering.
 - (c) Protein Engineering for Nonaqueous Solvents.
 - (d) Chromosomal Amplification/Gene Fusion.
 - (e) Hyperproduction and Secretion of Polyphenol Oxidase.
 - (f) Biological Separation of Phosphate from Ore.
 - (g) Catalysis-By-Design and Technology Transfer.
- (2) The Bioprocess Engineering work element emphasizes defining the basic engineering relationships between

molecular scale events and macro-level parameters. These parameters are required for designing scaled-up biocatalyzed chemical production processes and involve efforts in the design of novel bioreactor concepts that are likely to lead to substantially higher levels of reactor productivity, product yield and lower separation energetics. Additionally, results of work within this work element will be used to establish the technical feasibility of critical bioprocess monitoring and control subsystems. FY 1989 tasks included:

- (a) Immobilized Cell System for Continuous, Efficient Biocatalyzed Processes.
 - (b) Multimembrane Bioreactor for Chemical Production.
 - (c) Biocatalyzed Hydroxylation in Organic Solvents.
 - (d) Study on an Integrated Biological-Chemical Process for Continuous Production of Methyl Ethyl Ketone and 1,3 Butadiene.
 - (e) Separation by Reversible Chemical Association.
 - (f) Enzyme Catalysis in Non-aqueous Solvents.
 - (g) Immobilized Enzymes in Organic Solvents.
- (3) The Process Design and Analysis work element activities include energy-economic analysis of chemical and biocatalyzed processes; development of user-friendly software for process synthesis, integration and assessments; and conceptual process design and evaluation. Tasks for 1989 were:
- (a) Bioprocess Synthesis, Integration, and Analysis.
 - (b) Economic Evaluations of Potential Bioprocesses.

F. MANAGEMENT SUPPORT FUNCTION

The Management Support function has three areas of responsibility: Task Management and Planning, The Guidance and Evaluation Panel, and Industry Technology Transfer.

- (1) The Task Management and Planning area administers and coordinates the task elements of the Project. These responsibilities include: developing statements of work and the evaluation criteria for Project tasks; defining the sequence of accomplishments that will achieve Project objectives; monitoring and evaluating in-house and contract research progress;

compilation of quarterly reports, preparation and implementation of Annual Operating Plan (AOP), technical multi-year plans and budgets; personnel assignments; identifying new areas of research and issues to be studied; timely publication of Project research results and developments and their dissemination to DOE headquarters, industry and other researchers.

- (2) The Guidance and Evaluation Panel includes industrial and academic representatives who are leading authorities in the science and technology of biocatalysis. The panel is responsible for reviewing ongoing activities and future project plans as well as evaluating the Project's performance.
- (3) The Industry Technology Transfer task is to ensure strong, interactive relationships between the Biocatalysis Project and industry that will assist the transfer of the enabling technology required by the private sector for the production of chemicals via biocatalysis.

G. TASK DESCRIPTIONS

(1) Molecular Modeling and Applied Genetics

The Molecular Modeling and Applied Genetics work element consists of six research tasks: Theory of Biocatalysis/Biocatalysis by Design, Metabolic Engineering, Protein Engineering for Nonaqueous Solvents, Chromosomal Amplification/Gene Fusion, Hyperproduction and Secretion of Polyphenol Oxidase, Biological Separation of Phosphate from Ore and Catalysis-By-Design and Technology Transfer. The focus is on defining optimal microscale parameters for biocatalysts and developing practical applications of basic molecular biology research findings.

- (a) Theory of Biocatalysis (D.N. Beratan, Jet Propulsion Laboratory). The challenge of designing new catalysts based on known biocatalytic reactions requires an understanding of enzymatic catalysis at the molecular level. Achieving a quantitative understanding of catalysis is also one of the most important and fundamental problems in molecular biophysics. Without the presence of specific catalysts, nearly all of the biological chemical reactions with which we are familiar would not occur. Understanding the processes which control the reactivity and specificity of enzymatic reactions

will provide us with the knowledge needed to exploit similar mechanisms for technological applications.

The design of biocatalysts, or enzymes, poses a unique set of challenges compared to the design of small molecule catalysts. First, our understanding of the molecular details of biochemical reactions is in a more primitive state than our understanding of small molecule chemistry. Second, it is not yet clear a priori whether the surrounding protein superstructure plays an active or passive role in a particular biocatalytic reaction. For this study we have chosen the simplest biocatalytic reaction, electron transfer within cytochrome C and heme, as a basis for our theoretical and experimental studies. We propose to: a) map the electron transport routes ("tunneling") in enzymatic proteins, b) refine the design criteria for efficient charge separation in biocatalysts, c) perform experimental and theoretical studies on model separated charge systems for reiterative analysis, d) broaden the applicability of electron transfer theory, and e) design and synthesize catalysts to deliver electrons to active sites which will perform secondary catalytic reactions.

- (b) Metabolic Engineering (J.E. Bailey, California Institute of Technology). Metabolic engineering refers to the directed rearrangement or augmentation of metabolic pathways, using recombinant DNA techniques, in order to provide overproduction of metabolites or metabolic derivatives. Changing the enzyme makeup of the cell by genetic engineering will have secondary effects due to the cell's response to unnatural metabolic balance, often mediated through shifts in pathway branching and through ATP and reduced pyridine nucleotide levels. Experimental investigations of two different recombinant microbial systems with altered uptake and metabolic systems will be undertaken. In particular, the growth and phosphorous metabolism properties of Saccharomyces cerevisiae with enhanced glucose phosphorylating activity provided by plasmid-encoded proteins will be studied. Alteration in metabolism of Escherichia coli with amplified dihydrofolate reductase (DHFR) activity, again provided by expression of a plasmid gene, will be investigated. Finally, the aberration of introducing the Vitreoscilla

hemoglobin gene into E. coli will be studied.

The objectives of these experiments are to investigate the extent to which cellular metabolism is sensitive to amplification of individual activities in certain key positions in metabolism, to develop and demonstrate powerful experimental methods for investigation of metabolic perturbations (primarily ^{31}P in vivo NMR), to develop data for use in mathematical modeling studies of metabolic engineering, and to ascertain the effect of the Vitreoscilla hemoglobin gene on oxygen uptake, metabolism, and physiology in E. coli. Several different mathematical approaches to analysis of cell metabolism will be pursued as part of this research. A detailed single-cell kinetic model of recombinant E. coli will be enhanced to include more details of particular metabolic pathways, especially as pertaining to DHFR activity. In addition, global sensitivity analysis methods will be applied to explore those parameters in this model which are most important in simulated cell metabolism and growth kinetics. In addition, research will continue on mathematical frameworks for estimating a priori the overall cell response to metabolic engineering and for analyzing experimental data on altered metabolic pathway flows in a systematic, powerful fashion.

- (c) Protein Engineering for Nonaqueous Solvents (F.H. Arnold, California Institute of Technology). The ability to carry out biocatalysis in nonaqueous media greatly expands the potential scope and economic impact of biotechnology. The industrial applications of biocatalysts have been severely limited by constraints on the solvent environment of proteins, which normally require an aqueous medium for effective operation. With the advent of convenient methods for altering the amino acid composition and for synthesis of entirely new proteins, it is possible to engineer proteins that would be effective in nonaqueous solvents. However, the success of a rational design procedure for constructing proteins to use in organic solvents depends on understanding relationships among various factors: amino acid sequence, secondary and tertiary protein structure, and activity and stability in nonaqueous solvents.

The goal of this research is to begin to define

these relationships, by implementing an integral and iterative protein engineering approach based on information derived from studies of the model hydrophobic protein crambin. We propose to construct nonaqueous solvent-stable mutants of two extracellular proteases, subtilisin E and α -lytic protease. Alterations in the amino acid sequences of these enzymes will be made one-at-a-time, so that we can identify fundamental mechanisms by which one can expect to design and construct proteins that will function in polar nonaqueous solvents. Site-directed mutagenesis techniques will be used to test specific hypotheses concerning the role of surfaces' charges and internal crosslinks in maintaining protein stability in organic solvents. The random mutagenesis approach will be used to test whether alternative, non-obvious routes exist to stabilizing proteins in nonaqueous solvents.

- (d) Chromosomal Amplification/Gene Fusion (G. Bertani, Jet Propulsion Laboratory). Although genetic engineering techniques can be used to increase micro-organism productivity, e.g., to produce larger amounts of a chemical in a fermentation process, the genetic information (that results in higher production) may be lost as the microorganism multiplies. The objective of this activity is to determine if the desired genetically-engineered component (plasmid or associated gene) can be inserted into the chromosome and amplified in place. Since the desired genetic information (recombinant trait) would then be in the microorganism chromosome, it could not be easily lost as the microorganism multiplies. Thus the problem of plasmid stability could be addressed. Direct physical (as opposed to genetic) evidence for the insertion of the plasmid into the bacterial chromosome has been obtained.

The stability of bacterial strains resulting from this work will be compared with the stability of strains created by conventional plasmid splicing techniques. While laboratory strains of Escherichia coli are being used in this research, the general principles involved could easily be applied to other types of bacteria.

- (e) Hyperproduction and Secretion of Polyphenol Oxidase (W.V. Dashek and A.L. Williams, Atlanta University). Wood-decay within forests, a

renewable photosynthetic energy source, is caused primarily by Basidiomycetous fungi, e.g., white-rot fungi which possess the ability to degrade lignin, cellulose and hemicellulose, the main polymers of wood. In the case of at least one of these fungi, Coriolus versicolor, this ability results from the fungus' capacity to elaborate both extracellular cellulolytic and ligninolytic enzymes. Coriolus versicolor can synthesize and secrete these enzymes either in situ or in vitro. With regard to secretion, at least one of the enzymes, polyphenol oxidase (PPO) makes its appearance within a culture medium in a highly time-dependent fashion, i.e., extracellular enzyme begins to occur 7 days post-inoculation. Presumably, extracellular PPO originates via secretion of intracellular PPO. However, this presumption requires verification. Because PPO, an enzyme capable of converting diphenols to diquinones and oligomerizing syringic acid, appears to be inducible, it is conceivable that Coriolus versicolor could be a model for achieving over-production of enzymes. In addition, the system, which can be "scaled-up" to industrial levels of enzyme production, seems to be one in which the regulation of synthesis and/or secretion of both cellulolytic and ligninolytic enzymes can be investigated separately, i.e., experiments can be designed whose results may lead to the independent control of synthesis and/or secretion.

The objectives of this research are (i) to purify intracellular and extracellular polyphenol oxidases from Coriolus versicolor maintained in liquid culture and (ii) to determine if intracellular polyphenol oxidase is either de novo synthesized or activated. The approach is to (i) establish the time courses for the appearance of intracellular and extracellular oxidases and to subsequently determine specific activities, and (ii) to establish optimum growth conditions of the microorganism for maximum production of polyphenol oxidase, including determination of cofactor requirements.

- (f) Biological Separation of Phosphate from Ore (R.D. Rogers, Idaho National Engineering Laboratory). The phosphate industry utilizes about 0.3 quads/yr for the separation of phosphate from apatite ore. Therefore this task is directed toward development of bioprocessing for solubilization and separation of phosphate from ore. The specific objectives of

the proposed research are to: (i) define a microbiological system which will extract phosphate from its ore; (ii) develop a basic understanding of the biochemical mechanisms involved; and (iii) through the use of modern biotechnology develop a bioprocessing system for transfer to the phosphate industry. If a more efficient recovery process can be developed, it could also be applied to phosphate mine waste because ore containing <26% phosphorus is currently considered as mine backfill.

The approach will be to gain a thorough understanding of the biochemical interactions which cause the microbial release of phosphate from its ore. This will be accomplished by screening microorganisms obtained from areas of high phosphate content (i.e., phosphate mines, process waste streams, fertilized agricultural lands, etc.) for their ability to solubilize phosphate. Those organisms which are positive for the desired trait will be selected for further biochemical studies. When a better understanding of the mechanism of solubilization has been established, the work will be focused on development of methods to enhance production. Enhancement could be through physical/chemical stimulation, genetic manipulations or both. The next steps will be the gradual scale-up of the process. During each of these steps, evaluations of the most efficient engineering methodology will be made.

- (g) Catalysis-By-Design and Technology Transfer (L. Keay, Department of Energy). The objective of this effort was to determine the feasibility of expanding the molecular modelling effort. Four tasks were undertaken: (i) A Guidance and Evaluation Panel was convened to determine the appropriateness of a catalysis by design effort, (ii) A Research Opportunity Notice was published to determine industrial/academic interests, (iii) A series of workshops was organized to solicit information on the technical issues, and (iv) determining the future direction of the effort.

(2) **Bioprocess Engineering**

This work element consists of seven tasks: Immobilized Cell System for Continuous Efficient Biocatalyzed Processes, Multimembrane Bioreactor for Chemical Production, Biocatalyzed Hydroxylation in Organic

Solvents, Study on an Integrated Biological-Chemical Process for Continuous Production of Methyl Ethyl Ketone and 1,3 Butadiene, Separation by Reversible Chemical Association, Enzyme Catalysis in Nonaqueous Solvents, Immobilized Enzymes in Organic Solvents. They are primarily concerned with the definition of basic engineering relationships between molecular and micro-scale events and macro-level parameters, required to design and scale-up bioprocesses.

- (a) Immobilized Cell System for Continuous Efficient Biocatalyzed Processes (C.E. Scott, Oak Ridge National Laboratory). To decrease energy consumption and capital equipment costs, it is necessary to increase productivities of fermentation chemical processes significantly. Therefore, the goals of this task are to enhance productivity and operability of a fluidized bed reactor system containing immobilized microorganisms, and to investigate bioreactor dynamics, including the formulation and investigation of kinetic properties of biocatalyst particles, to lead to a better understanding of reactor behavior and control predictability. The reactor consists of a column containing immobilized cells; the substrate (glucose) and nutrients are passed into the bottom of the column and the product ethanol (in water) is continuously withdrawn from the top. The primary advantage of this type of bioreactor is increased rates of fermentation resulting from the high concentration of cells in the reactor.
- (b) Multimembrane Bioreactor for Chemical Production (M.L. Shuler, Cornell University). Immobilized cell reactors offer the potential to greatly improve volumetric productivities for bioreactors. An important form of such reactors is that of entrapping cells between membranes. Scale-up of such units can lead to problems with the transfer of sparingly soluble gases (e.g., O_2 and CO_2). These gas transfer problems can result in a loss of productivity due to starvation for gaseous nutrients or in the physical disruption of the membrane reactor due to pressure increases caused by incomplete removal of by-product gas (e.g., CO_2). Although membrane reactors are satisfactory for the retention of cells, the true advantages of membranes are incompletely utilized in current configurations. The use of membrane selectivity for in situ product recovery offers the potential to simplify process design and increase reactor

productivity by avoiding often encountered problems with product inhibition.

A multimembrane reactor concept is suggested. Four layers exist: one for cell entrapment, one for substrate flow, one for gas flow, and one for the flow of an extractant which selectively removes product. Task efforts include (i) development of a mathematical model relating the micro environment to reactor performance, (ii) development of a process simulator from the mathematical model, (iii) experiments to validate the model and to monitor the interrelationship of cell physiology to immobilization, and (iv) evaluation of long-term reactor operation.

- (c) Biocatalyzed Hydroxylation in Organic Solvents (A. Klibanov, Massachusetts Institute of Technology). The emerging field of enzymatic catalysis in non-aqueous media has the potential for making a profound impact on chemical processing. In order to fully utilize this potential, it is critical to rationally enhance and optimize the catalytic performance of biocatalysts in organic solvents. The objective of this task is to investigate an approach to controlling enzyme action in organic media which takes advantage of the high conformational rigidity of enzymes in anhydrous environments. An enzyme is lyophilized from an aqueous solution containing an appropriate ligand. The dry enzyme sample is subsequently washed with an anhydrous organic solvent to remove the ligand. The resultant enzyme, while in the dry state or in anhydrous organic solvents (but not in water), retains the conformation induced by the ligand, even after the ligand's removal. For example, subtilisin lyophilized in the presence of a ligand exhibits properties strikingly different from those of the same enzyme lyophilized under identical conditions but without ligand: enhanced catalytic activity, altered substrate specificity, and increased stability. We intend to mechanistically investigate this phenomenon and to explore its scope and generality. Our aim is to develop a rationale for altering at will characteristics of enzymes in organic solvents such as activity, selectivity, and stability by locking them in appropriate ligand-induced conformations. We will also explore the possibility of creating artificial receptors by lyophilizing theretofore inactive proteins in the presence of ligands, followed by washing out the

ligands. If these ligands are transition state analogs, then new biocatalysts in organic solvents will ensue. This study should afford rationally designed, superior biocatalysts in organic solvents and hence more efficient energy utilization in enzyme-catalyzed chemical processing by leading to shorter reaction times, more selective transformations, and more productive use of biocatalysts.

- (d) Study on an Integrated Biological-Chemical Process for Continuous Production of Methyl Ethyl Ketone and 1,3-Butadiene (G.T. Tsao, Purdue University). The objective of this task is to demonstrate the technical feasibility of an integrated Biological-Chemical process for the continuous production of two high volume/low value products: methyl ethyl ketone (MEK) and 1,3-butadiene from fermentable sugars. This conceptual bioprocess has been divided into two segments: production of 2,3-butanediol from fermentable sugars using an immobilized cell reactor containing living cells and selective adsorption of 2,3-butanediol from the aqueous fermentation broth using a solid adsorbent column.

Work will involve the analysis of production of optically active 2,3 butanediol in both stirred and air lift fermenters. Selective adsorption research involves the analysis of various materials as adsorbents.

- (e) Separation by Reversible Chemical Association (C.J. King, University of California, Berkeley). The objective of this task is to examine and evaluate the use of reversible chemical association, or complexation, with organic agents as a method for separating polar organic substances from dilute aqueous solutions, e.g., bioprocess product or waste streams. The goal is to obtain sufficient understanding of underlying chemical, equilibrium, and transport behavior to enable rational selection of separating agents, methods of regeneration, and methods of implementation, as well as rational conceptual design and economic evaluation.

In the production of carboxylic acids, processes such as fermentation yield low concentrations of carboxylic acids in an aqueous multicomponent solution. The subsequent separation, purification, and concentration of the carboxylic

acids are often difficult and energy intensive.

Data have been obtained for co-extraction of water when methyl isobutyl ketone (MiBK) is used as a diluent with Alamine 336 for extraction of succinic acid. Work is beginning on interpretation and modeling of co-extraction of water. Equilibrium data have been obtained for extraction of succinic acid by Alamine 336 with an alkane diluent. Now data for three diluents (the alkane, chloroform, and methyl isobutyl ketone) can be compared and interpreted. Work has been started on investigation of spectroscopic methods for interpretation of complexation in both carboxylic acid/amine and alcohol/phenolics extraction systems.

- (f) Enzyme Catalysis in Nonaqueous Solvents (H.W. Blanch, University of California, Berkeley). To permit the use of enzymes, in either free or immobilized form, to more broadly enter into the production of large scale chemicals, techniques for increasing the solubility and transport rate of organics at the site of enzyme action must be found. The approach proposed here is based on the use of a second organic liquid, in which the substrate or product is soluble to a much greater extent than in an aqueous system. The second organic liquid may be water miscible or immiscible. Such systems are used currently for the enzymatic transformation of steroids. A second aspect of the use of enzymes in nonaqueous environments is the potential to run many reactions "backwards". In cases where water is a reaction substrate, its high activity in aqueous solution generally results in a shift of equilibrium to favor the normal products of the reaction. In a non-aqueous system such reactions may be forced in the reverse direction, as water will be present at low concentrations. Examples of this type of reaction include esterification, peptide synthesis and dehydration reactions such as urea formation from $(\text{NH}_4)_2\text{CO}_3$.

In order to make the use of enzymes in non-aqueous environments a feasible possibility, several problems related to the nature of proteins must first be addressed. At low water concentrations, as are found with use of polar organic solvents, enzymes may undergo conformational transitions, which may lead to gross denaturation. Enzymes with disulfide bridges are less prone to such

transitions, and the use of detergents, which form ion-pairs with the enzyme, may also reduce the effect of conformational changes. Thus part of the proposed research and development effort will focus on understanding these aspects of protein chemistry.

Two model systems are being examined to develop generic technology for this effort. The first of these is the oxidation of the cholesterol in a two-phase system employing an organic solvent with the enzyme immobilized in an aqueous phase within microcapsules. The second model system to be examined is the conversion of ammonium pyruvate to tryptophan using the enzyme tryptophanase in a micellar or liquid membrane system where reactant and product are transferred by an ionic carrier. The key factors being addressed are the yields of product, rates of product formation and mass transfer rates of reactant to the catalytic site. These data will then permit an assessment to be made of the potential of these processes for further development and large-scale operation.

- (g) Immobilized Enzymes in Organic Solvents (H. Zemel, Allied-Signal). The application of enzymes in organic solvents to industrial processes has identified areas of research where difficulties might exist. These areas include water removal and control, enzyme deactivation by organic solvents, mass transfer restrictions, and the effect of continuous long term operation. The objective of this work is to investigate the physical chemistry of immobilized enzymes in organic solvents. The work will focus on the transesterification of steroids with subtilisin, and glycerides with lipases. Analytical methods will be developed which will describe the effect of water on the immobilized enzyme while in the organic solvent phase. These methods will also identify the optimal enzyme substrate concentrations as well as the optimal pH and solvent media.

(3) **Process Design and Analysis**

The Process Design and Analysis Work Element activities include energy-economic analysis of chemical and biocatalyzed processes; development of user-friendly software for process synthesis, integration and assessments; and conceptual process design and evaluation. There were two research tasks for 1989:

Bioprocess Synthesis, Integration, and Analysis; and
Economic Evaluations of Potential Bioprocesses.

- (a) Bioprocess Synthesis, Integration, and Analysis (J.D. Ingham and N.K. Rohatgi, Jet Propulsion Laboratory). Two candidate products, acetaldehyde and ethyl acetate, have been selected for detailed comparative assessments and process synthesis because the added value (relative to ethanol) exceeds 100%, and any additional energy for conversion of alcohol can be minimized by energy integration of process steps. Although computer codes exist that can be used to model and assess the efficiency and economics of bioprocesses, none are available that are generally applicable to many different processes and that quickly provide uniform, comparable assessments that account for many of the unusual characteristics of bioprocesses, or contain the needed bioprocess data bases, e.g., to include capabilities relevant to microscale behavior, kinetics, biomass pretreatment, high solids processing, and biocatalyst stability. Therefore, it has been proposed to initiate a collaborative effort with Solar Energy Research Institute (SERI) and industrial participants to modify and expand existing codes to develop a general bioprocess program that can be used for rapid energy-economic assessments of bioprocesses. These assessments will be used primarily to determine the benefits of proposed or realized technological advances, to define effective research directions and to evaluate the competitiveness of specific bioprocesses for the large-scale production of chemicals.

- (b) Economic Evaluations of Potential Bioprocesses (R.M. Busche, Bio-EGN Associates). The objective was to determine the economics of several bioprocess concepts to attempt to establish if they could lead to profitable commercial processes. (The National Corn Growers Association was a cosponsor).

The process concepts that were evaluated are: (i) n-butanol, where the products are extracted from the broth; (ii) recombinant aerobes for production of specialty chemicals; (iii) production of ethanol using Zymomonas mobilis or yeast; and (iv) extractive fermentation for production of acetic acid.

SECTION III

FY89 TASK ACCOMPLISHMENTS

Technical discussions included in this section were provided by the investigators. Because of this, a unique perspective of the authors' work is obtained.

A. Molecular Modeling and Applied Genetics

- (1) Theory of Biocatalysis (D. N. Beratan, Jet Propulsion Laboratory). The success of many biocatalytic processes is based on the efficient delivery of an electron to a specific catalytic site in a protein. It is essential that electrons not be delivered to inappropriate sites, as this may be toxic to the metabolic machinery. Biological electron transfer reactions involve charge transport over relatively large distances between weakly coupled sites. The intervening peptide medium provides this coupling so that rate of transfer is expected to be quite sensitive to details of this structure.

We are developing methods to predict how changes in amino acid residues (e.g., changing amino acid identity, disrupting hydrogen bonds, and altering van der Waals contacts) between donor and acceptor alter the electron transfer rates. We have implemented a survey method which uses the atomic positions and connectivities provided by the protein crystal structure to predict the pathways (combinations of interacting bonds in the protein) which couple donor to acceptor. The goal of this work is to provide design tools for biocatalysts which rely on electron transfer delivery to the active site.

The theoretical pathway predictions are being tested in genetically engineered and synthetically modified proteins synthesized in the Gray and Richards groups at the California Institute of Technology. Site-directed mutagenesis has been used so far to effect pathway modifications and provide attachment sites for redox probes (typically $\text{Ru}^{3+}(\text{NH}_3)_5$ bound to the histidine residue in the protein). Figure 3-1 shows pathways between the heme of cytochrome C and two histidine residues for which the Ru derivative has been made and the transfer rates measured (heme to Ru electron transfer). So far, the pathway calculations provide the most complete description of the transfer rates in this family of molecules, with observed rates

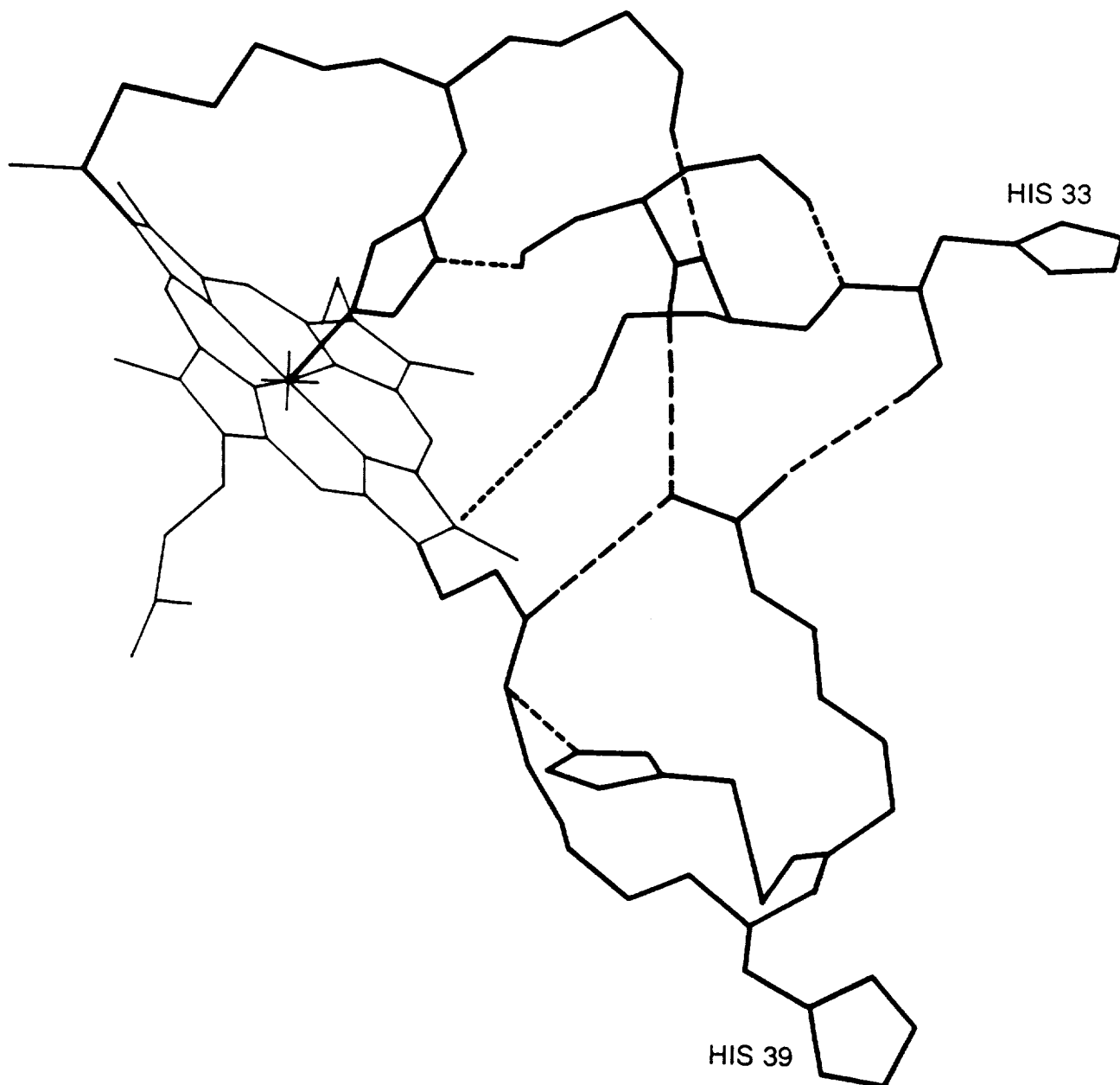


Figure 3-1. Yeast cytochrome C model. The pathways found for the His 33 and His 39 ruthenated derivatives of yeast cytochrome C are shown. The number of predicted pathways is rather small (dash lines). Synthetic modifications to the intervening groups in this protein could be used to modify the electron transfer rate and its temperature dependence.

correlating well with the "effective number" of bonds in the pathway. Improvements to the model will include corrections for interactions between multiple paths and corrections due to bond type differences. In parallel, a modest effort aimed at synthesizing small molecule electron transfer systems to aid in parameterization of the pathway model is beginning.

An intriguing spin-off idea for an ultra-small memory device arose from the above research. We realized that by using biological electron transfer chromophores and known strategies for tuning the rates of electron transfer, an ultra-small shift register memory device could be built. The information carrier in this device is the electron and the information would be processed using photoinduced electron transfer. We are now planning to embark on an experimental program to synthesize proof-of-concept structures for this device.

- (2) Metabolic Engineering (J. Bailey, California Institute of Technology). The primary focus of this work is the Vitreoscilla hemoglobin system and its applications in improving industrial organisms. Additional research has been conducted on Escherichia coli energetics and on metabolite measurement systems using nuclear magnetic resonance (NMR) spectroscopy. Another portion of this research has explored metabolic engineering of the ethanol pathway in yeast.

Earlier work in this project resulted in successful cloning and expression of the Vitreoscilla hemoglobin (VHb) gene in E. coli. Early studies demonstrated that respiration rates and cell growth under oxygen-limiting conditions were improved by the presence of hemoglobin. Further research has demonstrated enhanced growth from a variety of genetic constructs including two wild-type strains which have the Vitreoscilla hemoglobin gene inserted into the E. coli chromosome. These integrants express a sufficient quantity of hemoglobin to improve microaerobic cell growth, and provide hemoglobin-bearing hosts which can be used as a foundation for enhanced productivity. By inserting plasmids into these strains, it has been shown that the presence of hemoglobin significantly enhances cloned gene product accumulation under microaerobic conditions. This has been demonstrated both for cloned β -galactosidase and for cloned chloramphenicol acetyltransferase.

In order to clarify the biochemical role of cloned hemoglobin in altering cell function, its localization within E. coli and within Vitreoscilla has been determined. Approximately fifty percent of the active

protein resides in the cytoplasm, with the remainder found in the periplasm. A mathematical model including facilitated transport by hemoglobin of oxygen through the periplasm has been formulated and analyzed to indicate that hemoglobin should improve respiration rates by approximately ten percent in E. coli. The biochemical role of hemoglobin in the cytoplasm has not been determined conclusively, but may involve interaction with the respiratory apparatus of E. coli.

Initial studies of cloned Vitreoscilla hemoglobin in E. coli reveal that a promoter sequence from Vitreoscilla, naturally associated with the Vitreoscilla hemoglobin gene, had also been isolated. It has been clearly shown that this promoter is very sensitive to environmental oxygen in the expression of Vitreoscilla hemoglobin in E. coli. By removing the hemoglobin structural gene and fusing different reporter enzymes to the VHb promoter, we have shown that the promoter is responsive to oxygen at the level of transcription, and that hemoglobin itself is not required for regulation of this promoter. The promoter has the practically useful property of switching maximum activity when the oxygen content of the culture is low. Switching culture oxygen to low values is an extremely easy manipulation to implement in a bioreactor, given the large oxygen demand of the active cell mass. We have demonstrated in batch-fed fermentations using recombinant E. coli bearing plasmids which contain the VHb promoter fused to β -galactosidase and CAT genes that the promoter can be rapidly induced. An eleven-fold increase in activity, following rapid reduction of dissolved oxygen due to changes in aeration and agitation conditions, has been found. The VHb promoter system alone should provide a very useful regulated, portable promoter for expression of cloned enzymes and other proteins in E. coli.

Based upon a previously formulated mathematical model of the kinetics of the ethanol production pathway, sensitivity analyses suggested that ATP utilization rate and fructose-6-phosphate phosphorylation rate were two important control points for overall ethanol production. The predictions of the model have been verified by experimental studies in which ATPase activity was manipulated by adding a specific chemical inhibitor and phosphofructokinase (PFK) activity was modulated by inserting a cloned PFK gene into the cells. The changes in ethanol production rate resulting from decreases in ATP activity and increases in PFK activity were reasonably well anticipated in quantitative terms by the kinetic model.

An integral part of the analysis of these metabolically manipulated yeast strains was in vivo NMR spectroscopy to monitor intracellular metabolites and also to follow, simultaneously, consumption of glucose as well as ethanol and glycerol production. In these NMR experiments, we employed the custom dual resonance phosphorus-31-carbon-13 probe on our 300 MHz NMR spectrometer to accomplish time-resolved measurements of external and intracellular metabolites.

Anticipating the importance of in vivo NMR spectroscopy measurements of ATP levels and proton gradients across the cytoplasmic membrane of bacteria, studies were initiated on aeration of cells within the magnet of the NMR spectrometer. A model probe was constructed and instrumented to obtain mass transfer measurements in a previously reported airlift aeration design for the NMR probe. Based upon these measurements, it appears that satisfactory aeration of cultures can be achieved at higher cell densities, giving good NMR measurements. But control of foaming in these aerated systems requires additional effort. NMR measurements of intracellular metabolite levels and transmembrane pH differences have been utilized as a basis for a new mathematical model for ion transport through the E. coli cytoplasmic membrane.

- (3) Protein Engineering for Nonaqueous Solvents (F.H. Arnold, California Institute of Technology). The goal of this project has been to demonstrate that enzymes can be engineered at the level of the amino acid sequences to improve their stability in polar nonaqueous solvents. We are constructing nonaqueous solvent-stable enzymes by site-directed mutagenesis of natural enzymes, based on a rational set of design criteria developed during an earlier phase of this project.

Several "design rules" for protein stability in nonaqueous solvents were proposed, based on our understanding of solvent effects on the forces that contribute to the stability of folded proteins in solution and our studies of crambin, a natural protein that is stable and soluble in a wide variety of polar organic solvents. We have begun to test the proposed design rules by making selected alterations in the amino acid sequences of two bacterial proteases, subtilisin E and α -lytic protease. In addition to testing the design rules by site-directed mutagenesis on these two hydrolytic enzymes, we are also planning random mutagenic experiments combined with screening

the resulting mutants for improved stability. These studies will help us to identify additional mechanisms, not considered in our earlier work, by which stability in polar organic solvents can be achieved.

Proteases are enzymes which hydrolyze peptide bonds in aqueous solution. As with many other enzyme-catalyzed hydrolyses, peptide bond cleavage is not energetically favored over bond synthesis; the reaction proceeds towards hydrolysis because of the extremely high concentration of the reactant water in solution. In the absence of water, proteases will synthesize new peptide bonds from peptides or amino acids. These synthetic reactions can be carried out with the high specificities and stereoselectivities exhibited in aqueous media. Although the potential to carry out new chemistry and synthesize new materials using enzymes in organic solvents is very attractive, even relatively stable enzymes are quickly denatured in solvents of practical interest. This is not surprising since enzymes such as these proteases have evolved under selective pressure to function in primarily aqueous solution. In an organic solvent, a new balance of noncovalent forces will be found, and whether that new balance leads to disruptive conformational change depends strongly on the critical forces involved. From our studies of crambin and its water-soluble homologs, it was predicted that internal protein-protein hydrogen bonding would play an important role in maintaining protein stability in polar organic solvents. Therefore, an amino acid substitution that would lead to improved internal hydrogen bonding should have a beneficial effect on enzyme stability, provided that no other important interactions are disrupted by the changes. We also predicted that free charges on the protein surface, which are well-solvated in water, would become unfavorable in nonaqueous solvents and could possibly lead to conformational changes and loss of activity. Removing free charges from the protein surface by replacement with neutral amino acids should improve performance in nonaqueous media.

These two design rules have been tested by site-directed mutagenesis in the two proteases mentioned above. We have convenient *E. coli* expression systems for both subtilisin E and α -lytic protease, and all purifications and assay procedures have been worked out. Our first results have come from subtilisin E. An amino acid substitution which leads to improved hydrogen bonding results in a fifty-fold increase in the enzyme's stability in 40% dimethylformamide. A second substitution which replaces a surface charge

with a neutral residue also improves the stability, this time by a factor of 2. We have also determined that the effects of these two non-active site substitutions are additive: the subtilisin E variant containing both mutations is significantly more stable in DMF than either of the single mutants.

A major research goal for us has been to determine to what degree improvements in enzyme thermostability are correlated to improved stability in polar nonaqueous solvents. Much research has been carried out to determine mechanisms of protein thermostability, and this research has been translated into moderately effective mechanisms for engineering thermostability into enzymes. In this regard it is very interesting to note that the thermostability of one of the subtilisin E mutants (improved hydrogen bonding) is improved, while the second variant (modified surface charge) is even slightly less thermostable than the wild-type enzyme. Therefore, there is some overlap between mechanisms for improving thermostability and stability in nonaqueous solvents, but the overlap is by no means complete. One of our goals for future work is to define more precisely which mechanisms are likely to lead to improved stability in nonaqueous solvents and improved thermostability, and which mechanisms will have opposite effects on these two properties.

We anticipate that in the long term this research will provide us with clearly defined strategies for selecting enzymes that are suitable for use in polar organic solvents and for engineering enzymes to exhibit improved stability in such media.

- (4) Chromosomal Amplification/Gene Fusion (G. Bertani, Jet Propulsion Laboratory). During the year we have made progress in several directions. For reasons that will become obvious, we divide the material in two sections.

Externally controlled plasmid replication

This covers the work with strains carrying a ts-polA mutation. In these strains all derivatives of plasmid pBR322 are unable to replicate if the incubation temperature is above 37°C. We have previously reported the integration of our plasmid p240 and its amplification, in the absence of cytoplasmic copies, on the chromosome of such a strain. Those conclusions were based on 3 independent amplification events. In all these cases the amplified chromosomal segment included the whole plasmid.

Six additional amplification events were obtained and studied by the Southern blot technique (we have prepared the appropriate biotinylated probe, and can now avoid using radioactivity): two were obtained at 30°C, the others at 41°C. The duplicated segments again contained the whole plasmid, with the exception of one isolate where the amplification process occurred within the plasmid, and its precise map location is being studied. Since chromosomally amplified strains were obtained at both permissive and non-permissive temperature, one may conclude that plasmid replication does not interfere with, nor is required for, the amplification event.

As a control to some of our experiments a mutant resistant to high concentrations of tetracycline was isolated. While this strain can be as resistant to tetracycline as the amplified strains, it clearly grows less efficiently.

The replication (or attempts to replicate) of an integrated plasmid is thought to interfere with the replication of the host chromosome (Yamaguchi & Tomizawa, 1980, *Molec. Gen. Genetics* 178:525). The effect is probably conditioned by the position of the integration site on the chromosome and by the direction of replication of the plasmid with respect to that of the chromosome. The plasmid we have used to-date, when integrated, is co-directional in its replication with the chromosome. By appropriate *in vitro* cutting and joining we have isolated two derivatives of p240 which, when integrated, ought to have direction of replication contrary to that of the bacterial chromosome. One of these new constructs transforms recombinant deficient recipient strains at abnormally low frequencies and shows defective growth characteristics. This new finding is only of indirect interest for the project, but may be significant for our understanding of plasmid biology.

Self-regulated plasmid replication

The work reported above involved a strain conditionally defective for DNA polymerase I. This strain had been chosen at the start because it afforded a very practical method for the elimination of cytoplasmic plasmids through a temperature shift-up. The presence of the *polA* mutation however made the introduction of a *recA* mutation (to abolish recombination and thus stabilize chromosomally amplified strains) very problematic. An alternative route using strains with normal polymerase is desirable. Plasmids may be

eliminated by other methods. In fact, our first isolation of strains with a low antibiotic resistance level, interpreted as due to a single integrated plasmid copy, was based on selection in minimal growth medium at high temperatures, using strains with a normal polymerase. Selection in this situation is known to favor strains that have lost all plasmids. Our reinvestigation of those strains has led us to several new observations.

Apparently fitness in minimal media at high temperatures is affected not only by the presence of a plasmid, but also and, surprisingly, more strongly by the presence of the gene for tetracycline resistance.

We found that several of our isolates showing the level of resistance corresponding to a single copy of the tetracycline resistance gene per cell, still carried cytoplasmic plasmids. These plasmids however were not of the original type. They had presumably lost by deletion the gene specifying tetracycline resistance. Furthermore, on shifting these strains to higher concentrations of tetracycline, new spontaneous plasmid constructs, often of relatively large size, appeared. These are probably the result of interactions between cytoplasmic plasmids, having lost their tetracycline resistance gene, and the chromosomally integrated copy, still carrying such gene. One might expect some of these larger plasmids to carry fragments of the bacterial chromosome that normally is adjacent to the prophage attachment site.

A particularly interesting case was the observation of isolates carrying cytoplasmic plasmids that consisted of repeats of a small segment of the original plasmid (presumably including the origin of replication, but not the tetracycline resistance gene). Different isolates differed in the number of repeats forming the individual plasmids, while the repeat, as judged by size after digestion with specific restriction enzymes, appeared to be always the same. In one particular isolate, there were no demonstrable cytoplasmic plasmids and the level of resistance to tetracycline was typical of a single gene copy, however, the presence of a short sequence of the original plasmid in many copies could be demonstrated in Southern blots. Apparently, this sequence had been incorporated into the bacterial chromosome. When this isolate was subcultured in high tetracycline concentrations, a derivative could be obtained which had reformed cytoplasmic plasmids, presumably through some form of excision of the complete copy carried on the

chromosome. These new results bring to light previously unsuspected interactions between cytoplasmic plasmids and the bacterial chromosome.

- (5) Hyperproduction and Secretion of Polyphenol Oxidase (W.V. Dashek and A.L. Williams, Atlanta University). The objectives of this project are: i) to develop reliable and rapid methods for the purification of polyphenol oxidase, ii) to determine whether extracellular polyphenol oxidase results from either de novo synthesis or activation, iii) to enhance polyphenol oxidase synthesis by substrate induction, mutant production and/or cloning, iv) to develop methods for the "batch" culturing of Coriolus versicolor, v) to establish the route of polyphenol oxidase secretion. These objectives are being examined by combined electron microscopy, biochemistry, immunochemistry and molecular genetics.

Last year, we reported the time-dependent appearances of both intracellular and extracellular PPO, the substrate specificity of extracellular PPO, the de novo synthesis of intracellular PPO and the partial purification of extracellular PPO. In addition, application of recombinant DNA techniques to the C. versicolor system revealed that: 1) genomic DNA could be isolated and purified by CsCl₂-ethidium bromide gradients, 2) numerous DNA fragments could be generated when genomic DNA was subjected to EcoRI digestion, 3) an EcoRI-generated fragment could be inserted into the EcoRI site of the chloramphenicol resistance gene of plasmid pBR325, and 4) Escherichia coli cells transformed with recombinant plasmid were resistant to ampicillin and tetracycline but sensitive to chloramphenicol. Both poly-(A)⁺ and (A)⁻-RNA were isolated and purified for use in in vitro translation to obtain cell-free synthesis of PPO.

During the past year, we have focused on the enhancement of C. versicolor's PPO gene expression as well as assessing the route of and regulating PPO secretion.

Enhancement of C. versicolor's PPO Gene Expression by Recombinant DNA Technologies

Additional efforts were devoted to "scaling-up" and over-producing PPO from C. versicolor. Specifically, recombinant DNA technologies were utilized to generate putative clones harboring the PPO gene(s) by both the DNA shotgun and cDNA approaches. The cloned insert(s) were successfully recovered from vectors (i.e., pBR325

or gt-11) by the use of selective restriction enzymes. Subsequently, the size of the insert (0.6 kb) was determined by gel analysis and a partial restriction map was constructed. Additionally, PPO activity was detected in extracts of *E. coli* Hb101 transformants. Also, PPO activity was assayed in nitrosoquanidine-treated and non-treated filtrates of *C. versicolor*. Moreover, it appeared that these recombinant plasmids (pcV series) expressed PPO activity in *E. coli* cells in liquid media. Currently, experiments are under way to modify, truncate or alter these cloned DNA insert(s) for enhanced expression capabilities with respect to PPO production.

Finally, we propose to develop and optimize fermentation processes conducive to the expression of recombinant proteins in microbes. Specifically, *E. coli* transformants and/or phage lysogens carrying non-mutagenized and mutagenized cloned inserts will be screened for their abilities to express the incorporated *C. versicolor* PPO gene(s) for the mass production of PPO itself.

Enhancement of *C. versicolor*'s PPO Secretion

With regard to PPO secretion, experiments were performed to ascertain whether secretion was mediated by subcellular organelles. To this end, combined electron microscopical and biochemical techniques were employed. Whereas the microscopy involved the ultrastructural cytochemical localization of PPO within growing hyphae, the biochemistry centered about coupled cell fractionation and assay of PPO within resultant isolated organelles. For the localization, it was established that PPO activity could be detected within homogenates of *C. versicolor* hyphae which had been pre-fixed 30 min in 2.5% glutaraldehyde buffered with 0.1 M, pH 7.4 cacodylate. Control hyphae were suspended in cacodylate buffer only for the same time period as those in buffered glutaraldehyde. Therefore, intact hyphae were pre-fixed in buffered glutaraldehyde as above, washed with cacodylate, incubated 18 hr with 50 mg dihydroxyphenylalanine (control lacked dihydroxyphenylalanine) for 18 hr at 0°C and then 1 hr at 37°C, post-fixed for 20 min in 2%, cacodylate-buffered OsO₄, washed with cacodylate, dehydrated through a graded acetone series and embedded in Spurr's reagent for electron microscopy. At the present time, plastic-embedded hyphae which were incubated either with or without dihydroxyphenylalanine are being sectioned for the electron microscopical localization of PPO within hyphal organelles.

In addition, preliminary attempts have been initiated to localize PPO within hyphae via immunogold labeling procedures. To accomplish this, the purity of Sigma (St. Louis, MO) mushroom PPO was examined by gel filtration on Sephadex G-150, Sephadex DEAE-ASO ion exchange chromatography and Laemmli SDS-PAGE. As the results derived from the application of these techniques indicated that Sigma's PPO was homogenous, the enzyme is being utilized as an antigen in the immunization of rabbits for antibody production and the subsequent labeling of affinity chromatography-purified antibody with gold. Immunogold labeling will serve as an additional electron microscopical procedure to localize PPO within hyphae.

Another approach to establish the route of secretion has involved following time-dependent alterations in PPO specific activity in isolated hyphal subcellular organelles. To this end, hyphae cultured for 0,3,6,9,12 and 15 days were harvested, sonicated into buffered-sucrose and subsequent to withholding aliquots of the sonicates for total protein and PPO assays, the sonicates were subjected to 500, 1,000, 10,000, 40,000 and 105,000 x g centrifugations. Then, the distributions of both 280 nm absorbing substances and PPO within organellar fractions were determined. The results of these experiments suggested that PPO synthesis occurred early (day 3-4) in a 16 day time course for the in vitro growth of the organism and that secretion was mediated by organelles since a low and constant PPO specific activity was observed in the cytosol. The identities of the organelles participating in secretion are being examined by marker enzyme analysis and electron microscopy. When hyphae were homogenized with a mortar and pestle rather than sonicated, the PPO within the organelles was eliminated and the intracellular PPO was recovered in the cytosol. This may provide a means of purifying intracellular PPO from organellar membrane proteins which could also serve as a source of antigens in the preparation of PPO antibodies.

As for the regulation of PPO secretion, experiments have been initiated to enhance the elaboration of PPO into the external milieu. These experiments involve the employment of respiration inhibitors in an effort to block PPO secretion without inhibiting synthesis, i.e., is there an inhibitor concentration which affects only secretion? With this in mind, sodium azide and sodium fluoride in growth medium were added (control: medium addition only) to 4 day-old cultures

(prior to PPO secretion) and both mycelia and growth medium harvested at 16 days of culture. At the present time, both intracellular and extracellular 280 nm absorbing substances' contents and the specific activities of PPO, intracellular succinate dehydrogenase and O_2 consumption (which is a measure of hypha respiration) are being quantified.

As for the application of genetically-engineered wood-degrading enzymes, they may be employed to remove "unwanted" lignocellulosic substances in the paper-pulp industry and to render certain agricultural commodities, e.g., straw, more digestible to ruminants. Further, an available supply of ligno-cellulolytic enzymes could be of marked industrial value. To maintain an adequate supply, substrate induction coupled to hyphal, "batch culture" and genetic engineering technology is sought to overproduce PPO in keeping with the overall mission of this project.

- (6) Biological Separation of Phosphate from Ore (R.D. Rogers, Idaho National Engineering Laboratory). Applying biotechnology to the process of solubilizing phosphate ore has been divided into the following seven tasks: (1) selection and culturing of those microorganisms from the environment which can produce soluble phosphate from insoluble sources; (2) determination of the biochemical phenomenon responsible for the solubilization process; (3) calculation of process kinetics and determination of rate limiting steps; (4) enhancement of the solubilization process of select organisms through the manipulation of external physical/chemical factors and internal genetic composition; (5) investigation of the possibility of using biomechanisms for the concentration/separation of soluble phosphate from process solutions; (6) evaluation and selection of a bioprocessing methodology which will maximize the peculiar phosphate solubilizing biochemistry of selected organisms; and (7) collaboration with industry in the cost-shared scale-up and integration of developed methodology into their phosphate extraction process stream. We are currently working on tasks 2, 3, 5, 6, and 7.

Two microorganisms (1 bacterium and 1 fungus) have been used for phosphate (PO_4) solubilization studies during the year. During this time both the bacterium E37 and the fungus I5G were tentatively identified as Pseudomonas cepacia and Penicillium funiculosum respectively.

The biological mechanism responsible for the

solubilization of the Idaho rock phosphate ore (RP) was investigated. Several references have been made in the literature which indicate that microbiologically produced organic acids are responsible for the solubilization of RP. It has been shown that the two RP solubilizing microorganisms used in this study produce predominately gluconic and 2-ketogluconic acids when grown in the presence of glucose. Based on the literature reports it was assumed that these two organic acids were responsible for RP solubilization. To test this theory, several carbon sources which were known not to produce gluconic or 2-ketogluconic acids during metabolism by microorganisms were substituted for the standard glucose in RP solubilization studies.

Results of these studies showed that the bacterium E37 was capable of solubilizing RP when grown in several of the carbon sources while the fungus I5G was only effective when grown with glucose and ethanol. Gluconic and 2-ketogluconic acids were not detected in the presence of any of the carbon sources though different organic acids were found with some of the carbon sources. These data could indicate that there are RP solubilizing mechanisms in addition to organic acid. Work is continuing on this aspect of RP solubilization.

Presently, a continuous bioprocessing system is being developed to help maximize the peculiar RP solubilizing biochemistry of the bacterium E37. A prototype of this system consists of a 2-liter stirred tank and a series of 125 mL secondary biocontact reactors. The concept behind this design is to provide conditions for the continuous production of biochemically derived compounds from E37. These biocatalytic compounds are then provided under steady flow conditions to the secondary biocontact reactors which contain RP. This setup allows for the study of RP solubilization under either steady state or varying conditions. Effluent from the contact reactors is analyzed for soluble PO_4 content and the total RP solubilized is calculated.

Use of the continuous system has resulted in a ten-fold increase in the quantity of RP ore being processed. The present system has an 85% efficiency over a 12 day period. It is expected that with optimization, the continuous system will be able to operate with increased efficiency at more concentrated pulp density (currently 1%) and with reduced processing.

In addition to the use of secondary biocontact reactors, effluent from the microorganism propagator is

being applied to RP contained in columns. This process simulates use of the microorganism under in situ conditions. Data from these studies are not yet available.

The FMC Corporation, Phosphate Chemicals Division, continues to be a direct contributor to this work both intellectually and monetarily through a technology transfer agreement.

- (7) Catalysis-By-Design and Technology Transfer (L. Keay, Department of Energy). In 1987, the Guidance and Evaluation Panel recommended that the areas of Catalysis-by-Design and Biocatalysis-by-Design should be studied in more depth, and a higher proportion of funds should be allocated to these areas.

It was therefore decided to solicit proposals in the areas of catalysis-by-design and biocatalysis-by-design, and a Research Opportunity Notice was published. The response was greater than expected with 47% of the responses from universities, 36% from R&D and chemical firms and 17% from research institutes and government. The responses, especially from the commercial sector, were insufficient to provide the in-depth information necessary to develop a research focus in the two design areas.

Another major point of concern in the program has always been that of technology transfer. It is clear that the United States still maintains a superior position in scientific research, but appears to lack the ability to successfully commercialize the results. It has been pointed out that much academic research is carried out and then attempts are made to interest the private sector in using the results. In the area of catalysis-by-design, it was decided to seek industrial input into the research proposals so that when the results became available, they would be the results that had been requested by industry. It was also decided that a cooperative working arrangement was more acceptable to the Department of Energy National Laboratories than to the academic community, and so it was proposed that the national laboratories be solicited for proposals under a National Laboratory/Industry Initiative on "Catalysis-by-Design".

A series of workshops was organized by Pacific Northwest Laboratories and held in 1989, with representatives invited from each national laboratory and a number of private companies, plus a few guest

speakers. The first was held at Caltech in July, 1989, on "Biocatalysis-by-Design", and was chaired by Dr. William Goddard of Caltech. The objective was to decide on specific technical issues for a solicitation of proposals.

The second workshop was held at Argonne National Laboratory in August, 1989, and was chaired by Dr. Alan Wilkes, Allied-Signal Corporation, the topic being "Chemical Catalysis-by-Design". The third workshop was held in Washington, and was on "Government/Industry Relationships". It was chaired by Dr. Ed David, former President of Exxon Research, and Science Adviser to President Nixon. The objective of this workshop was to explore whether industry would work with the Department of Energy and the National Laboratories, and determine how the problems of confidentiality, patents, etc., should be handled.

The workshops were attended by representatives from many large chemical and oil companies. The outcome was an agreement that a combined research in catalysis-by-design was desirable, and that companies would be willing to participate in cooperative proposals.

In December, 1989, a request for proposals was sent to the DOE national laboratories, soliciting proposals in two areas, "Catalysis-by-Design" and "Biocatalysis-by-Design", with the requirement that they have an industrial partner who would fund their share of the research and take part in this cooperative working agreement.

B. Bioprocess Engineering

- (1) Immobilized Cell System for Continuous, Efficient Biocatalyzed Processes (C.D. Scott, Oak Ridge National Laboratory). The ECUT Biocatalysis Program at ORNL seeks to increase fundamental and practical knowledge in the areas of advanced bioreactor concepts, efficient immobilized biocatalyst systems, and enzymatic catalysis in organic media. Ethanol fermentation has been enhanced in the three-phase immobilized cell fluidized-bed bioreactor (FBR). The use of high productivity FBR's is being extended to the production of neutral solvents and novel four-phase systems with simultaneous reaction and separation. Significant hydrodynamic differences have been observed between these systems with near-neutral solids and literature systems with high-density particles.

Integrated Fermentation and Separation

FBR's have the advantage that additional solid phases can be added to perform useful functions and that these phases will separate or stratify based on density or size. A novel system is being tested with two solids; one containing the immobilized fermenting microorganism and the other an adsorbent for the desired product. This concept for a biparticle FBR in either countercurrent or cocurrent operation will have ultimate use as a combined fermentation-separation system. Successful initial tests were made of the biparticle FBR for countercurrent operation that demonstrated the hydrodynamic feasibility. The particle density was controlled by adding specified amounts of inorganic oxide to 4% k-carrageenan gel beads that were 1.5 mm dia. Heavier particles were continuously added to the top of a fluidized bed to simulate countercurrent operation. The heavier particles (simulating the adsorbent particles) passed through the bed and were removed completely from the system. Feasibility tests of this reactor design showed complete separation of 1.07 and 1.09 g/L size particles.

Quantitative tests on separation rates have been made for countercurrent operation with and without gas flow in the liquid-fluidized bed. As expected, gas flow decreases the rate at which solids can travel through the bed. Successful preliminary tests were performed for cocurrent operation with a bed of particles of 1.07 g/L and lighter particles of 1.04 g/L density added to the bottom of the column and removed from the top respectively.

Lactic acid production was chosen as the initial test system. Several adsorbents have been tested and activated carbon was selected for the initial lactic acid adsorbent.

FBR Hydrodynamics and Modeling

The study of hydrodynamics and kinetics of the three-phase FBR is necessary in order to understand, model and scale-up systems with low density colloids. These multiphase systems are poorly understood and further complicated by gas production during fermentation. A computer program has been written to simulate reaction and axial dispersion in a columnar reactor with immobilized cells for the case of first order kinetics and is now being modified to more complex and realistic kinetics. This program will be used to analyze

previous experimental results of the immobilized cell FBR for ethanol fermentation. Experimental observations using a nonintrusive conductivity technique were made of the axial dispersion and the phase holdup of a three-phase FBR in large scale (up to 7.6 cm dia) columnar reactors. These experiments showed significant differences from studies in narrower columns (2.54 cm dia). In particular, the bubbles reached a stable size: ≈ 4 mm in the larger column, while in the narrower columns the bubbles coalesced to span the entire column (>2.5 cm dia).

Solvent Fermentation with Immobilized Cells

A bioconversion of continuing interest is the anaerobic fermentation of sugars into organic solvents such as acetone and butanol by Clostridia. A difficulty of the continuous use of these microorganisms is the need for an anaerobic environment. Cell membrane fragments that will reduce oxygen to water have been developed at ORNL. These fragments can be immobilized in gel beads along with microorganisms to create an anaerobic microenvironment within the biocatalyst despite the external conditions.

Cells of Clostridium acetobutylicum have been successfully immobilized with the anaerobic membrane fragments in gel beads of carrageenan and used to produce solvents from dextrose in batch experiments. Experimentation continued on the acetone-butanol fermentation with the use of another strain of Clostridium acetobutylicum with higher butanol and biomass yields. An immobilized cell FBR with Clostridium acetobutylicum was operated continuously for the acetone-butanol fermentation. The products, in order of decreasing effluent concentration, were butanol, acetone, acetic acid, butyric acid and ethanol. Anaerobic biocatalyst beads were successfully used for the production of the desired solvents and sustained the reaction in spite of the use of oxygenated feeds.

Enzymes in Organic Media

The use of enzymes bound to a polymer-dye complex increases both the solubility and the activity of several enzymes, such as cytochrome C, in organic media. Experiments confirming this were repeated. Tests of enzymatic activity, in organic media, against recalcitrant compounds were begun.

- (2) Multimembrane Bioreactor for Chemical Production (M. Shuler, Cornell University). The purpose of this project has been to test the feasibility of using a highly compartmented bioreactor for chemical production. Initial studies have focused on ethanol production from yeast. The original reactor concept consisted of four compartments separated by membranes. The gas layer is separated from the cell layer by a hydrophilic membrane; and the nutrient layer is separated from an extracting solvent (typically tributyl phosphate, TBP) by another hydrophobic membrane. The inclusion of the gas layer allows the efficient removal of CO₂ generated in the fermentation which has been a problem with most immobilized cell reactors. The hydrophilic membrane is used to entrap the yeast cells so as to increase effective cell density and volumetric productivity. The nutrient layer/solvent hydrophobic membrane allows the *in situ* extraction of ethanol into the TBP. By keeping the pressure of the nutrient layer higher than that of the solvent layer, solvent emulsification can be prevented. Prevention of emulsification protects the cells from the toxic effects of TBP seen in shake flask cultures. Droplets of emulsified TBP can interact adversely with the yeast cell surface.

Although the original concept has proved technically feasible, the current high cost of membranes and slow rates of diffusion of substrate and product within the cell layer would make its employment economically impractical for lower cost products. We have modified the system to reduce costs and increase reaction rates. The primary feature of this reactor system is four compartments, but compartmentation can be achieved without the use of membranes. Currently the membrane separating the gas and cell layer has been removed and replaced with a conductance-liquid-level control system. By cycling gas pressure we can alternately push liquid out of the cell layer and then pull it in through the hydrophilic membrane separating the cell layer from the nutrient layer. The liquid level as sensed by these conductance probes controls the switching of gas pressure. This convective flow greatly increases the rate of production of ethanol per unit membrane area.

By using this type of reactor we have achieved sustained continuous production of ethanol for over 3000 hours. In this recycle mode 80% of the 350 g/L glucose substrate stream was converted (e.g. approaching CFSTR behavior). Operation was stable with no reduction in performance over the last 2600 h of the

experiment. The apparent volumetric productivity was about 5 g/L-h based on the whole volume of the reactor, but the volume of the reactor system is arbitrary (e.g. the spacing of distances for nutrient, gas, and solvent flows was not optimized). Unlike other immobilized cell systems this reactor includes volume for product separation as well as formation.

When a corn starch hydrolysate was produced enzymatically in our laboratory and substituted for glucose, the reactor performance was essentially unchanged. Molasses-based medium could not be used because it fouled the membranes.

Reactor performance was compared using Saccharomyces cerevisiae and Zymomonas mobilis. The reactor performed better with the yeast. With Z. mobilis filamentation occurred. The filamentous mat prevented good volume exchange during pressure cycling, leading to the accumulation of inhibitory levels of ethanol in the cell layer.

To apply such a system on a commercial scale will require greater optimization and prediction of response to a wide range of operating scenarios. We have completed construction and verification of a reactor model that uses a structured, non-segregated model of the yeast population. The model predictions of reactor performance agree closely with experiment. The model predicts the effects of improved volume exchange (e.g. 0.45 μm pore membrane instead of 0.22 μm pore membrane) on productivity (about two-fold), improved solvents (two- to three-fold for a 10-fold increase in distribution coefficient) and increases in nutrient/solvent membrane area (about two-fold increase in productivity for six-fold increase in solvent/nutrient layer membrane area). The use of the 0.45 μm membrane is feasible for a commercial scale plug flow reactor system (PFR). While the increase in solvent/nutrient membrane area is feasible the increased productivity may not justify the extra membrane cost. The use of an improved solvent is an attractive option, but no such solvent has been identified. Finally, the model was used to predict performance when three recycle reactors were placed in series. The model predicts better than 98% conversion of an initial 200 g/L glucose feed, with near theoretical yields, and a volumetric productivity of about 5.5 g/L-h compared to 5 g/L-h for single stage unit at the same point in time with 350 g/L glucose feed and 80% conversion. These model simulations will provide a basis for an economic analysis of the reactor

potential.

- (3) Biocatalyzed Hydroxylation in Organic Solvents (A.M. Klibanov, Massachusetts Institute of Technology).
During the past year, our studies of biocatalysis in non-aqueous media have proceeded in two directions: a) development of a colorimetric method for the enzymatic analysis of gases, namely the determination of ethanol and formaldehyde vapors using solid alcohol oxidase; and b) design of novel receptors (selective adsorbents) by molecular imprinting of proteins. Below, we outline our major research accomplishments to date.

We had previously demonstrated that yeast alcohol oxidase, in addition to its ability to function as a catalyst in organic solvents, could also work in the absence of any liquid phase, i.e., catalyze gas phase reactions. Operational parameters of this system had been quantitatively examined. On the basis of this investigation, we have now developed a new enzymatic approach to the direct determination of ethanol vapors in the gas phase. The system is composed of alcohol oxidase, horseradish peroxidase, and the color indicator 2,6-dichloroindophenol dispersed on microcrystalline cellulose (avicel). Simple devices have been developed for the semiquantitative determination of ethanol in the breath. The devices are designed to produce a sharp color change at a set time of 1 minute (kinetic method) or a stable final color after 5 minutes (equilibrium method) for ethanol concentrations above the legal limit for driving. Such color changes are readily detectable by visual observation. Using standard plastic sheets for thin-layer chromatography and a transmittance densitometer, the system can also be used as a quantitative method for the determination of ethanol or formaldehyde (suspected human carcinogen) vapors. It was concluded that dehydrated immobilized enzymes may be useful for the analysis of hazardous gases in industry.

In the course of our investigations of enzymatic catalysis in organic solvents we realized that a profound novel feature of enzymes in an anhydrous milieu is their high conformational rigidity (manifested in such new characteristics as greatly enhanced thermostability and also ligand "memory"). We have now utilized the phenomenon of drastically lowered protein flexibility in anhydrous media (compared to water) to transform common proteins into selective artificial receptors in organic solvents. When a protein is dissolved in a concentrated aqueous solution of a polyfunctional organic compound, freeze-

dried, and washed with an anhydrous organic solvent to remove the ligand, the resultant "imprinted" protein has been found to bind much more of the template compound in anhydrous solvents than the non-imprinted protein in the same solvent (and both proteins in water). For example, bovine serum albumin imprinted with p-hydroxybenzoic acid binds 6.6 mole-equivalents of this ligand in diisopropyl ether, whereas the non-imprinted protein bound only 0.7 mole-equivalents. Even more striking results were obtained with another polyfunctional ligand, L-tartaric acid: 30 mole-equivalents of the ligand bound in ethyl acetate to the imprinted bovine serum albumin vs. 1 mole-equivalent for the non-imprinted one. Not only does imprinting convert the heretofore inactive protein into a potent receptor, but it does so with a marked selectivity: e.g., removal of both hydroxyl groups from tartaric acid (to yield succinic acid) results in less than half of the binding of this ligand (compared to tartaric acid) to albumin imprinted with tartaric acid; esterification or replacement of the carboxyl groups in tartaric acid with hydroxyls (to yield dimethyl tartrate and threitol, respectively) eliminates all appreciable binding.

The imprinting phenomenon has been demonstrated for several unrelated proteins (hemoglobin, lysozyme, and chymotrypsin, but not for poly-L-lysine, which is lacking the tertiary structure) and in different anhydrous organic solvents. It has been found that addition of water to the anhydrous organic solvents erases protein's "memory" by diminishing, and eventually abolishing, the preferential binding of the template ligand.

Imprinted protein-based receptors may prove to be useful for separations, purifications, or biosensors. This approach may also lead to the design of new artificial catalysts, e.g., if the template ligand is a transition state analog (as with catalytic antibodies) or the product of a condensation reaction, the resultant receptor may accelerate the reaction between substrates due to a proximity/orientation effect. Such studies are in progress.

- (4) Study on an Integrated Biological-Chemical Process for Continuous Production of Methyl Ethyl Ketone and 1, 3-Butadiene (G.T. Tsao, Purdue University). A continuous fermentation process for the production of 2,3-butanediol has been developed employing immobilized living cells. As shown in Figure 3-2A, a reasonable stable continuous run was maintained for over 30 days.

The immobilized cell beads are very stable. Some turbidity is observed in the exit stream, indicating loss of cells. However, new growth is apparently sufficient to maintain the continuous reactor in operation for a long period of time. It is important to determine how cells are dislodged from the beads, and how cells are replenished. To assist in these determinations we are now examining individual beads and trying to determine the cell distribution in a single bead. Knowing the growth kinetics and other rate processes, a mathematical model has been formulated to describe the single bead and overall reactor characteristics.

Meanwhile, the physiological state of the cells is also being investigated and a mathematical model for the cells has been developed based on the concept of internal balance of bioenergetics. In essence, from experimental results, we know that the cells will produce different types of products depending on the oxygen supply. Acetic acid would be the main product when the oxygen supply rate is high. Ethanol will be the main product under nearly anaerobic conditions. The yield of 2,3-butanediol is the highest with a limited, but controlled oxygen supply. The bio-oxidation affects the generation of NADH_2 and ATP, while each of the product formations will require different levels of energy. Therefore, through carbon balancing and the bioenergetics balancing, we have been able to model cell behavior. One of the important practical objectives of this effort is to use the model to help us carry out the transition from the initial batch operation, when the operation is just started, to a continuous steady state operation without upsetting the process (e.g., by wash out, oscillation, etc.). The transition protocol developed through the modelling effort was successfully applied. As shown in Figure 3-2B, the transition period with large fluctuations lasted about 4 hours. Even though the value of $q\text{O}_2$ still increased somewhat after that for a period of 16 additional hours, the value of $q\text{CO}_2$ was steady. About 20 hours after initiation of the transition protocol, the continuous operation became very stable.

- (5) Separation by Reversible Chemical Association (C.J. King, University of California, Berkeley). Reversible chemical complexation is a potentially attractive method for recovery of carboxylic acids, alcohols, glycols and related substances from the dilute and complex solutions which result from biomass fermentations. We have focussed on complexation

through extraction and sorption processes. Separations by complexation can also be carried out through extractive distillation, membrane processes, adductive crystallization, and foam and bubble processes.

Separation of Carboxylic Acids

Precipitation due to Stripping of Co-extracted Water.

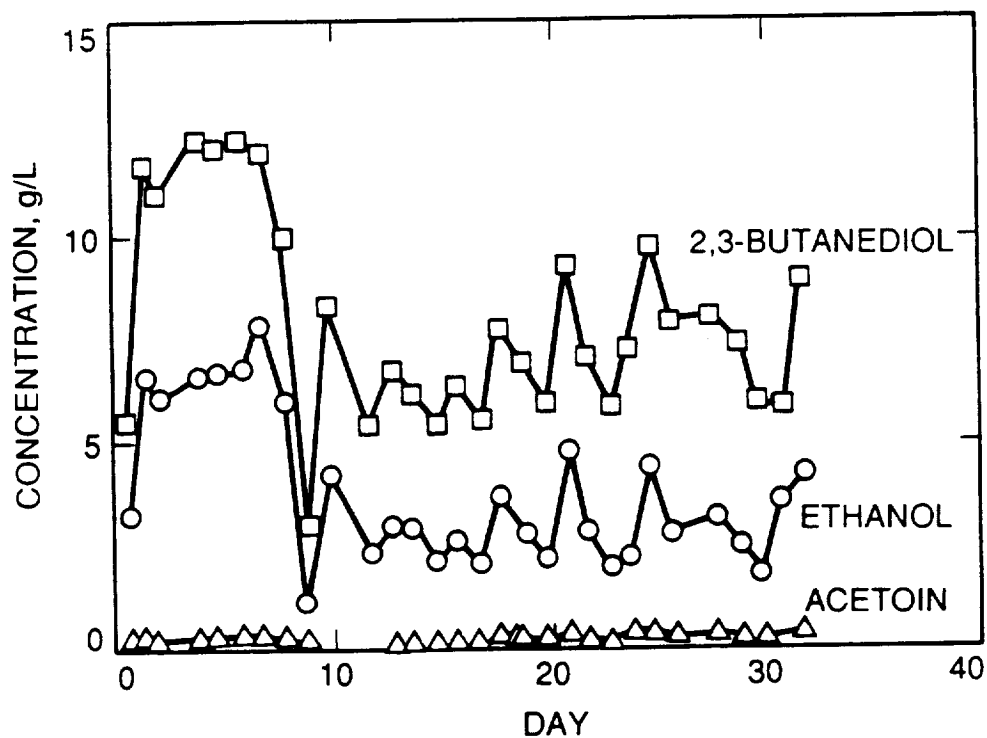
The presence of co-extracted water considerably increases the solubilities of carboxylic acids in certain organic solvents. This phenomenon forms the basis for a novel regeneration process for extracts containing relatively insoluble carboxylic acids. In this approach (1) a solvent is chosen in which this effect occurs and in which water is preferentially volatile, (2) co-extracted water is removed from the extract by stripping, and (3) the carboxylic acid product simultaneously precipitates and is thereby recovered.

The solubility of fumaric acid in methyl isobutyl ketone (MiBK) is increased five-fold in the presence of water. Positive effects of co-extracted water on fumaric acid solubility have also been found with n-octanol, n-butyl acetate, di-n-butyl ether, tributyl phosphate, and Alamine 336 in either MiBK or Toluene. The effect is non-existent or negative for tricetyl phosphine oxide, n-heptane, chloroform, and Alamine 336 in chloroform. Co-extracted water also increases the solubilities of succinic and adipic acids in MiBK. We are seeking to understand and capitalize further upon this phenomenon.

The equilibrium distribution ratios (K_d) for extraction of fumaric and adipic acids into MiBK at high dilution are 2.2 and 0.8, respectively. Thus MiBK extraction with regeneration by precipitation, concomitant with stripping of water, can be attractive for these acids. Cyclohexanone exhibits higher capacities for extraction of carboxylic acids than does MiBK. We are therefore exploring equilibrium distribution ratios and effects of co-extracted water on solubilities of carboxylic acids in cyclohexanone.

Back Extraction with an Aqueous Solution of a Volatile Tertiary Amine. Another promising method of regeneration involves back extraction of a carboxylic acid from an organic-phase extract (e.g., Alamine 336, a high-molecular-weight tertiary amine, in an organic diluent) into an aqueous solution of a low-molecular-weight tertiary amine (e.g., trimethylamine, TMA). The resultant aqueous solution is concentrated by

(a)



(b)

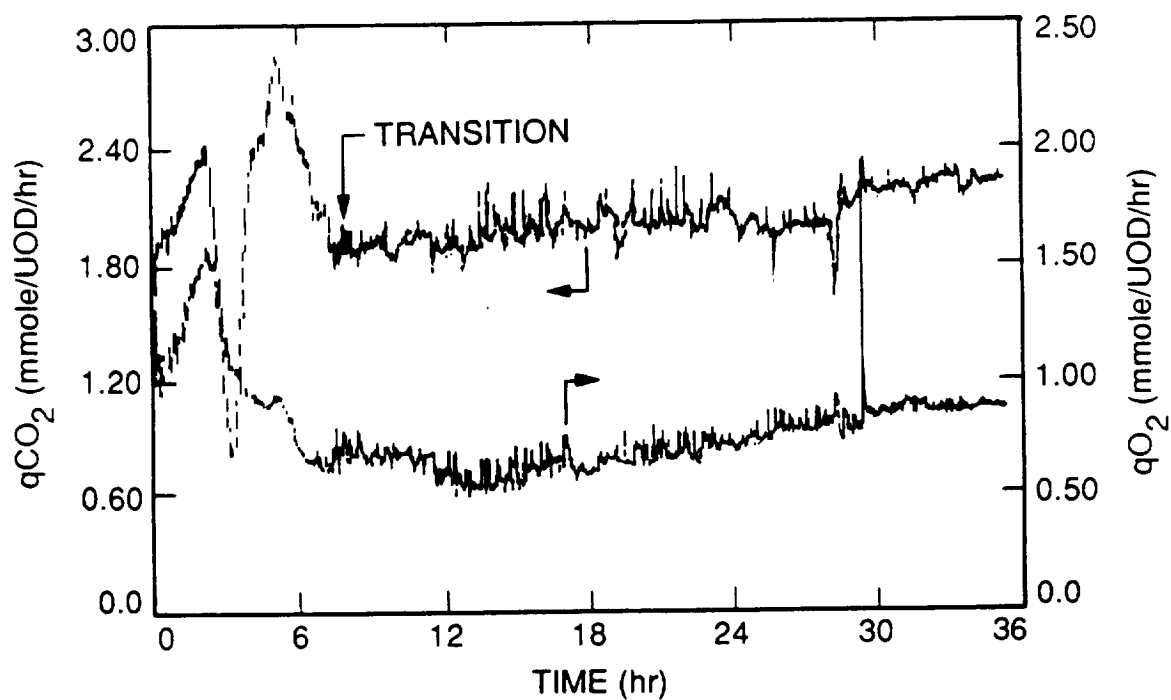


Figure 3-2. Immobilized cell bioreactor. (a) Long term continuous fermentation in an immobilized cell bioreactor. (b) Specific gas rates through bioreactor transition phase.

evaporation and the trialkylammonium carboxylate is decomposed thermally, yielding the volatile tertiary amine for recycle, as well as the carboxylic acid product. An aqueous solution of a tertiary amine is required since ammonia and primary and secondary amines are susceptible to amide formation upon heating with carboxylic acids. The high-molecular-weight amine has very low solubility in water, while the low-molecular-weight amine has high aqueous solubility.

Experimental results for back-extraction of lactic, fumaric and succinic acids from Alamine 336/MiBK extracts into aqueous TMA solutions have shown that there is strong ion pairing in the aqueous phase; i.e., essentially all the TMA pairs with carboxylic acid, even if this leaves very little carboxylic acid in the organic phase. Furthermore, partitioning of TMA into the organic phase is very low, as long as there is not a substantial stoichiometric excess of TMA. Thus the volatility and strong aqueous basicity provided by TMA are highly synergistic. The back-extraction results agree with a predictive model based upon measured values of K_d for the forward extraction and literature data for Pka 's of TMA and the carboxylic acids.

Aqueous back-extracts containing TMA and lactic, succinic and fumaric acids have been evaporated, with temperature and rates of evolution of water and TMA being monitored. In the cases of fumaric and succinic acids, crystals of the acid form in a viscous solution. It has been found effective to redissolve this mixture in MiBK and then evaporate MiBK under vacuum. This approach provides over 80% recovery of the carboxylic acid as crystals. Redissolution in MiBK probably allows more time for crystal growth in a solution of lower viscosity. By virtue of its much higher solubility in water, lactic acid does not form crystals and yields only a glassy mass, still containing TMA.

IR spectroscopic data have been obtained for solutions of succinic, fumaric and maleic acids in Alamine 336 solvent mixtures. The results reveal overloading tendencies and self-association, and are consistent with the earlier interpretation that we have made of extraction data for those species.

Adsorption. We are commencing research to develop attractive methods of regeneration for adsorbents laden with carboxylic acids. Methods of interest include solvent leaching, thermal swing, leaching with an aqueous solution of a low-molecular-weight tertiary

amine, and the use of a separable displacing acid.

Separations of Glycols

Borates complex reversibly with cis-diols. This phenomenon is used for analytical chromatographic separation of diols and sugars. When the -OH groups are adjacent, as in 1,2-butanediol, the complex forms a relatively stable five-member ring. Separation of the -OH groups by one carbon, as for 1,3-butanediol, gives a six-member ring which typically has a lower stability constant. 1,4-Butanediol would complex as a seven-member ring, which has little or no stability. Thus complexation with borates affords a way to fractionate among glycol isomers.

pH is important, since it is the boronate ion rather than the free boronic acid which forms the complex. The pK_a of boric acid is 9.2; hence boric acid complexes with diols are formed only at pH above 8. For recovery of glycols from solutions of neutral or lower pH, a boronic acid with higher acidity is necessary.

Aryl boronic acids are less soluble in water than is boric acid and form stronger complexes with diols than does boric acid. Furthermore, appropriate substitution of a phenylboronic acid can increase acidity. 3-Nitrophenylboronic acid (NPBA) has pK_a near 7 and forms complexes with diols at pH as low as 5. We have determined that the solubility of NPBA in water is 0.40 wt%.

Stability constants have been measured for 1,2-propanediol with 3-nitrophenylboronic acid, by means of the Antikainen method, wherein the apparent pK_a is measured as a function of diol concentration. Measurements will also be made for other diols.

In an extraction mode, one can use ion pair extraction of the complexed boronate. One approach to be explored will be to extract with NPBA and a quaternary amine in an organic diluent.

Regeneration methods are needed. Measurements will be made to assess the relative attractiveness of swings of temperature, pH, and diluent composition.

Liaisons

We are working collaboratively with Charles D. Scott, Brian Davison, and associates at Oak Ridge National

Laboratory to identify sorbents which are attractive for in situ use in the ORNL continuous, fluidized-bed fermentation process. The Principal Investigator is also a member of the Research Advisory Committee for the Michigan Biotechnology Institute, which has a major program to develop technology for commercial production of carboxylic acids and derivative products from biomass.

- (6) Enzyme Catalysis in Non-aqueous Solvents (H.W. Blanch, University of California, Berkeley). This research program has been concerned with developing techniques which will permit the wider use of enzymes in the synthesis of organic chemicals. Although enzyme catalysis offers unique features, such as stereo- and regiospecificity, and the ability to conduct the reactions at ambient temperatures and pressures, it has not found widespread use in the chemical process industry, due primarily to the low aqueous solubility of many potential reaction substrates of interest and the cofactor regeneration requirements of many oxidation-reduction enzymes. In order to increase the range of possible substrates, the research has examined the use of two-phase aqueous-organic systems. Two-phase aqueous-organic systems maintain the enzyme and cofactors in an aqueous miniphase, and the substrate in a second organic phase which enhances its solubility. Several approaches have been examined, including the use of liquid membrane systems, reverse micelles and microcapsules. Several model enzyme reactions have been studied: the production of tryptophan from indole, the oxidation of the steroid cholesterol, cofactor requiring alcohol dehydrogenase and the protease chymotrypsin.

We have examined the use of liquid membranes to provide an aqueous miniphase. Here the enzyme is retained within an inner aqueous phase surrounded by a thin spherical layer of a second organic phase. This is then placed in a second aqueous phase. By selecting appropriate phase-transfer catalysts (e.g., liquid ion exchangers), substrates and products can be partitioned from one phase to the other. A second system examined is the use of reverse micelles. A micelle is formed upon the addition of a small volume of water to a much larger volume of immiscible organic solvent containing a surface active agent. The surfactant is oriented at the interface of the two phases so that the hydrophilic head is in contact with the aqueous phase and the hydrophobic tail is in the bulk organic phase. Micelles are thus usually envisioned as spherical clusters of surfactant surrounding an inner pool of

water, which is capable of solubilizing large hydrophilic molecules such as proteins. An enzyme solubilized in the inner water pool is thus protected from direct contact with the surrounding organic phase. Favorable partition coefficients for the enzyme substrates and products can change the equilibrium concentrations by several orders of magnitude, due to the driving force created by the free energy change of the transfer of a reagent from one phase to another. This approach to enzyme catalysis in organic solvents thus appears most attractive and of considerable generality.

We have also examined encapsulation as a method for protecting an enzyme, overcoming some of the problems associated with recovery and recycle of micelles and providing a means for developing a fluidized bed bioreactor based on encapsulated enzymes. In addition, microencapsulation provides a promising approach for developing a robust method for cofactor recycle. The microcapsule may contain several enzymes and thus provide a relatively simple mechanism for regeneration of cofactors such as NAD^+ that are required in many oxidation and reduction reactions of commercial significance. We describe such a system in the following sections.

Reactions in Reverse Micelles

Previous work on the use of reverse micelles employed tryptophanase in the synthesis of tryptophan. This system, while attractive from an industrial point of view, was difficult to study at the molecular level because of its complexity. The current research employs simpler systems whose physical-chemical nature may be more easily probed. The enzymes currently being investigated are alcohol dehydrogenase and chymotrypsin. To avoid the use of cosurfactants, other systems are being studied including anionic, cationic and nonionic surfactants as well as single and double tailed surfactants.

Horse liver alcohol dehydrogenase (HLADH) was chosen as a model enzyme since its structure is known and EPR spin-labelling techniques are available to probe the active site. HLADH is an oxidoreductase which can catalyze the production of many organic chemicals, in addition to flavour and fragrance compounds. It employs the cofactor NAD^+ , as is the case with many potentially industrially significant enzymes. Reverse micelles may provide an environment in which cofactor regeneration is possible. Kinetic studies on a model

system, cinnamaldehyde production, have been performed under a variety of reverse micelle conditions (see figure 3.3A). A maximum rate of reaction with w_0 , the water to surfactant ratio, has been observed in several reports, in some instances with rates higher than those found in aqueous systems. EPR is being used to determine if structural changes to the enzymes are responsible for these kinetic results.

Chymotrypsin is a small, well-characterized enzyme that may be used for dipeptide synthesis. A variety of EPR spin-labelling techniques are available for this enzyme. Kinetic studies are currently under way for this enzyme.

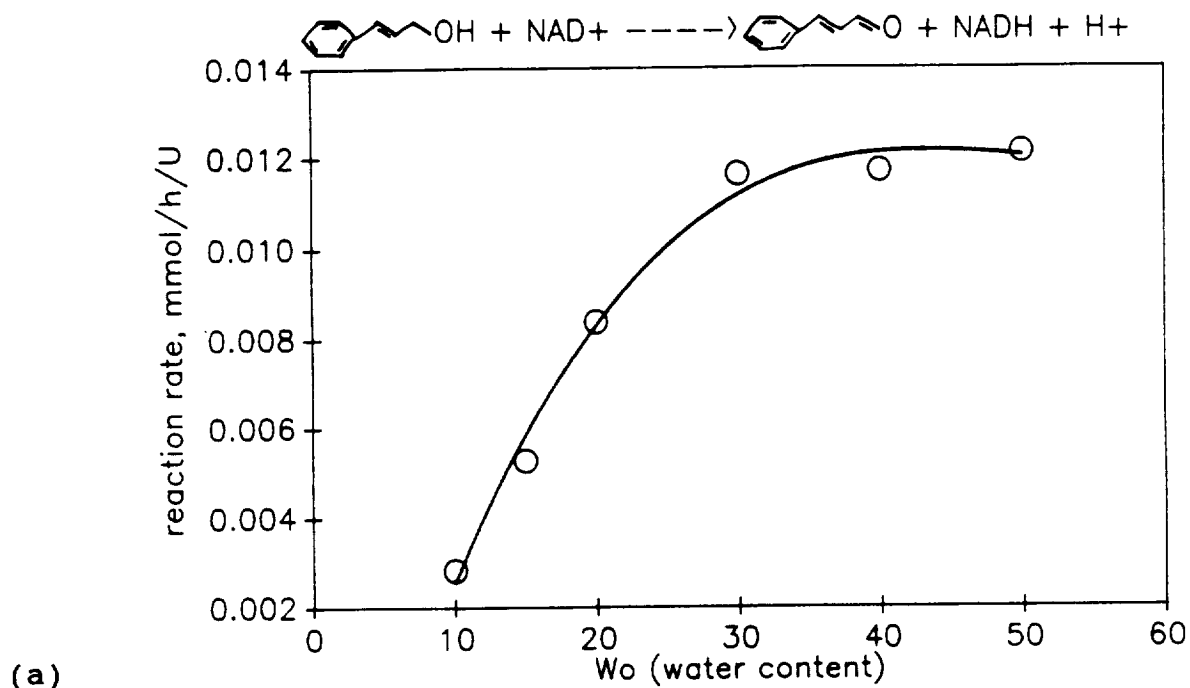
Various surfactants were employed with different hydrophilic head groups. The anionic surfactant chosen for study was dioctyl sulfosuccinate (AOT). AOT has been extensively studied in reverse micelle systems and the wide range of water contents available makes it especially attractive. Furthermore, no cosurfactant is necessary with AOT. Cetyltrimethylammonium bromide (CTAB) was used as a cationic surfactant. Like AOT, CTAB can also solubilize varying amounts of water. Other cationic surfactants are currently under investigation. Unlike CTAB, these surfactants are double-tailed which facilitates reverse micelle formation.

Light scattering is a powerful tool which can be used to ascertain the structure of reverse micelles. Dynamic light scattering measurements determine the hydrodynamic radii of the reverse micelles. The distribution of reverse micelle sizes has been determined using a continuous size distribution analysis program. Static (classical) light scattering measurements determine the apparent micellar molecular weight. From these molecular weights, aggregation numbers can be calculated.

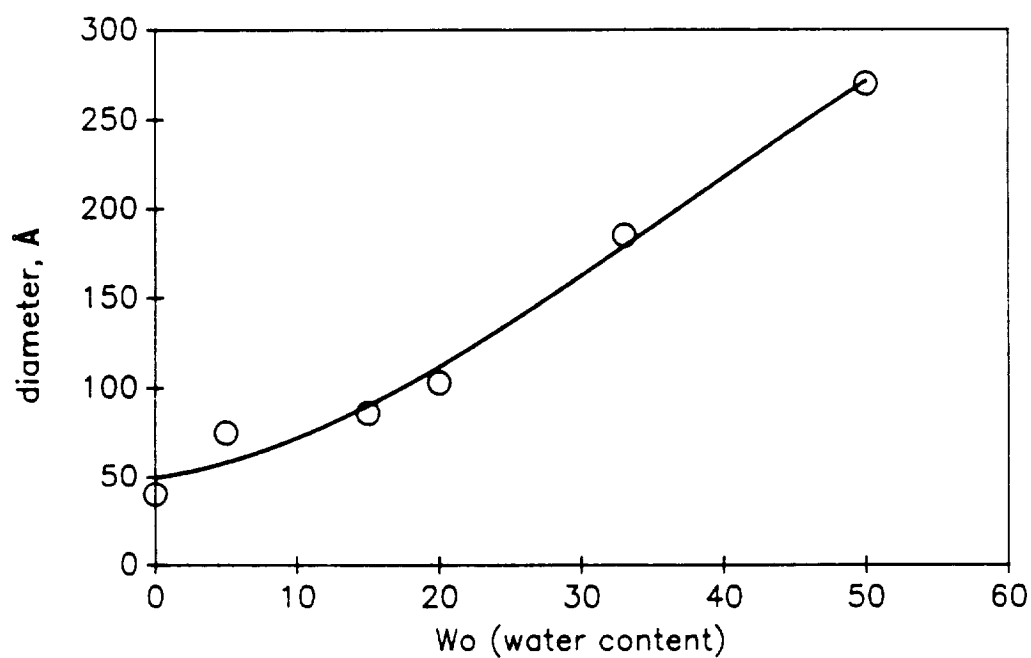
Dynamic light scattering results for the AOT iso-octane system are shown in the figure 3.3B. These results are used in conjunction with the kinetic results to further characterize the system.

Reactions in Microcapsule Systems

The oxidation of cinnamyl alcohol, catalyzed by yeast alcohol dehydrogenase (YADH), was studied in a microcapsule-organic solvent system. The enzyme reaction requires cofactor NAD^+ , which must be used in catalytic amounts and recycled because of its high



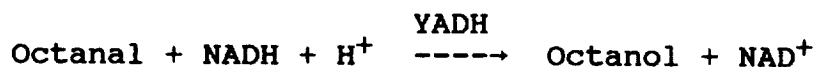
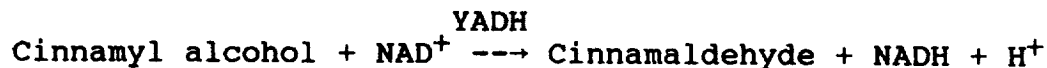
(a)



(b)

Figure 3-3. AOT/isocatane reverse micelles. a) Effect of water content on reaction rate. Reaction mixture: 0.1 mM cinnamyl alcohol, 0.25 mM NAD⁺, pH 8.5, enzyme activity in aqueous solution 0.032 mmol/h/U. b) Effect of water content on micelle size.

cost. In this work we have employed a second substrate of YADH, octanal, to regenerate NAD^+ during the reaction. The coupled-substrate reaction is as follows:



Equilibrium favors cinnamyl alcohol oxidation ($K_r = 100$); with excess octanal (40 mM), one hundred percent conversion of cinnamyl alcohol (10 mM) can be obtained within a few hours.

Batch reactions were conducted with encapsulated YADH/ NAD^+ dispersed in cyclohexane containing cinnamyl alcohol and octanal. Batch initial rates were linear in enzyme concentration within the capsule and independent of interfacial area per volume capsule (Figure 3-4), thus confirming that mass transfer does not influence the reaction rate in this system. The interfacial area of capsules was varied by changing the capsule diameter. Encapsulated YADH exhibited Michaelis-Menten kinetics with respect to cinnamyl alcohol concentration in the organic phase. The stability of encapsulated YADH/ NAD^+ stored in cyclohexane was satisfactory ($t_{1/2} \approx 5$ days). Hemoglobin (5 wt %) was found to be very important for maintaining catalytic activity in the microcapsule system and the two-phase dispersion.

The kinetics of encapsulated YADH/ NAD^+ were also studied in a continuous-flow reactor. Because of capsule fragility, these studies were conducted in an expanded/fluidized bed reactor. With cyclohexane as the continuous phase, the capsule bed expanded, but did not fluidize. A carbon tetrachloride/cyclohexane mixture (27%/73%, $p=0.99$ where $p_{\text{cap}} \approx 1.1$ g/mL), however, could fluidize microcapsules within the range of superficial velocities available in the current experimental system.

The operational stability of encapsulated YADH/ NAD^+ and the effect of residence time (flow rate) on conversion of cinnamyl alcohol were investigated in the continuous-flow system. The catalytic half-life in the reactor was unsatisfactory (5 hours) compared to the storage stability, due to enzyme deactivation by the product cinnamaldehyde. To determine the effect of

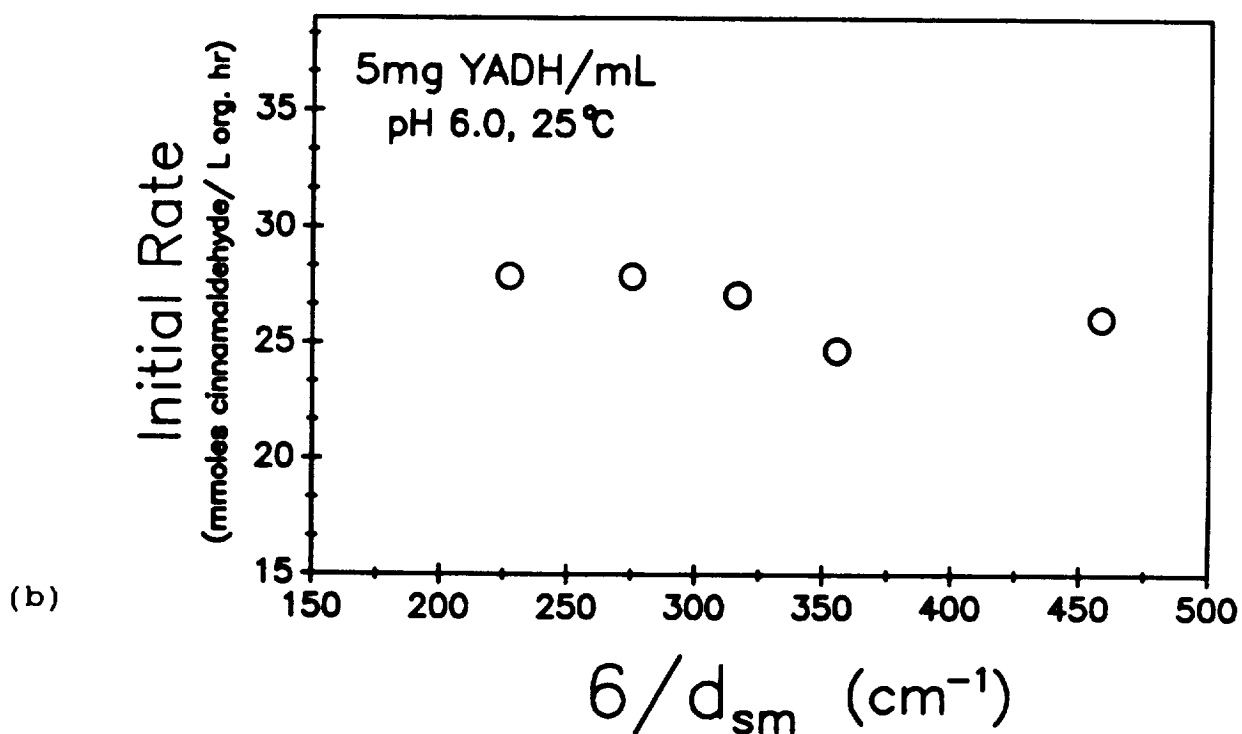
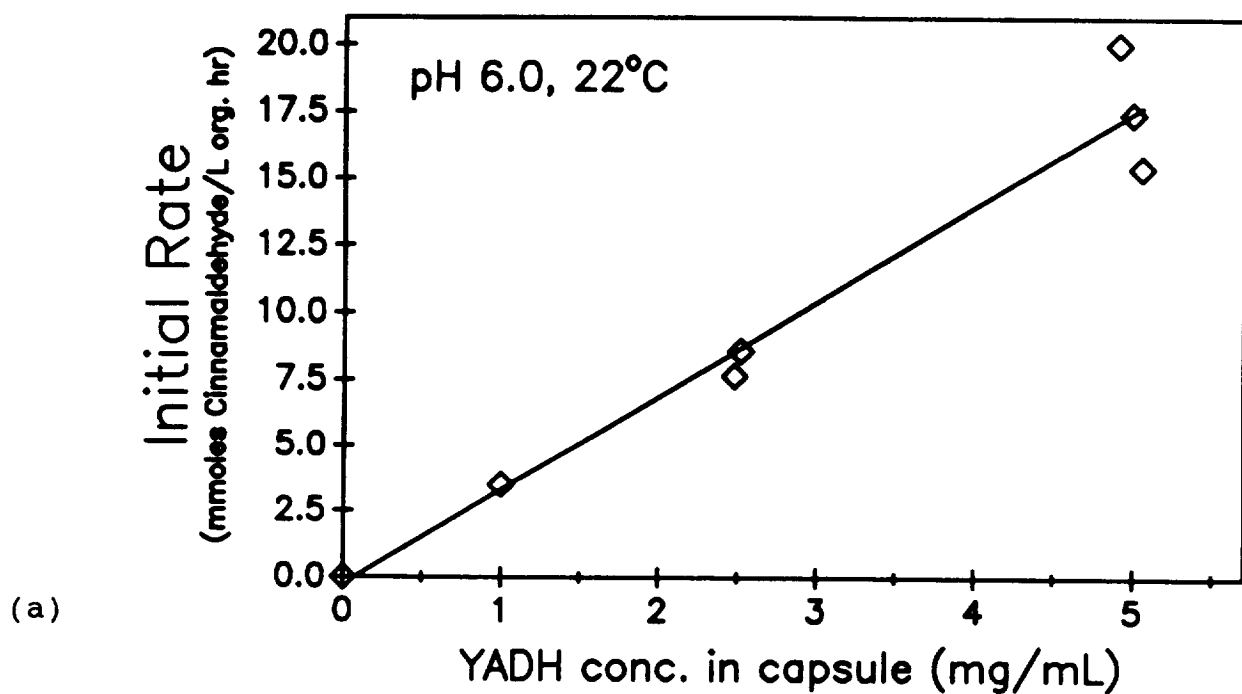


Figure 3-4. Micelle enzyme kinetics. The effect of (a) enzyme concentration and (b) interfacial area on the rate of reaction.

residence time on conversion, the flow rate was changed and the steady-state conversion of cinnamyl alcohol measured. Conversions were corrected for inactivation during the course of the experiment and they are plotted in Figure 3-5A as a function of residence time for different conditions. The axial dispersion in our experimental system was measured and found to be small enough ($D/UL \approx 0.1-0.2$) to assume plug flow. Figure 3-5B demonstrates that the kinetics of encapsulated YADH in the expanded and fluidized bed can be predicted quite well using batch kinetics and the plug-flow performance equation.

- (7) Immobilized Enzymes in Organic Solvents (H. Zemel, Allied-Signal Research). The objective of this project is to investigate the physical chemistry of immobilized enzymes operating in organic solvents and use the knowledge obtained to increase the efficiency of transesterification processes catalyzed by immobilized enzymes in nonaqueous media. The work performed in the first 6 months has laid the foundations for the project. The first step was to identify a suitable model system, namely, an enzymatic transesterification reaction which has commercial potential. We have examined two systems: stereoselective transesterification of steroids and interesterification of triglycerides. In both cases the enzymes involved have been reported to operate in organic media. We were successful in observing both reactions take place and after developing equally good analytical methodology for the two, have settled on the lipase-catalyzed triglyceride interesterification reaction due to its larger commercial appeal and its higher efficiency. We are focusing on one particular application of this system: the production of low-cost alternatives to cocoa butter from inexpensive palm oil. However, there are many other possible commercial applications of an efficient enzymatic interesterification reactor.

The next step was to optimize conditions for the reaction. Using the rate of stearic acid incorporation into tripalmitin as a measure of reaction progress, we have screened lipases from eight different sources for their activity. Six out of the eight exhibited various degrees of activity with two performing an order of magnitude or better than the others. One of the two, the Amano Lipase P from Pseudomonas cepicia was selected for further optimization. A support for the immobilized lipase was chosen out of a number of incompressible inorganic supports which would be useful in an industrial process. In general, activity in the

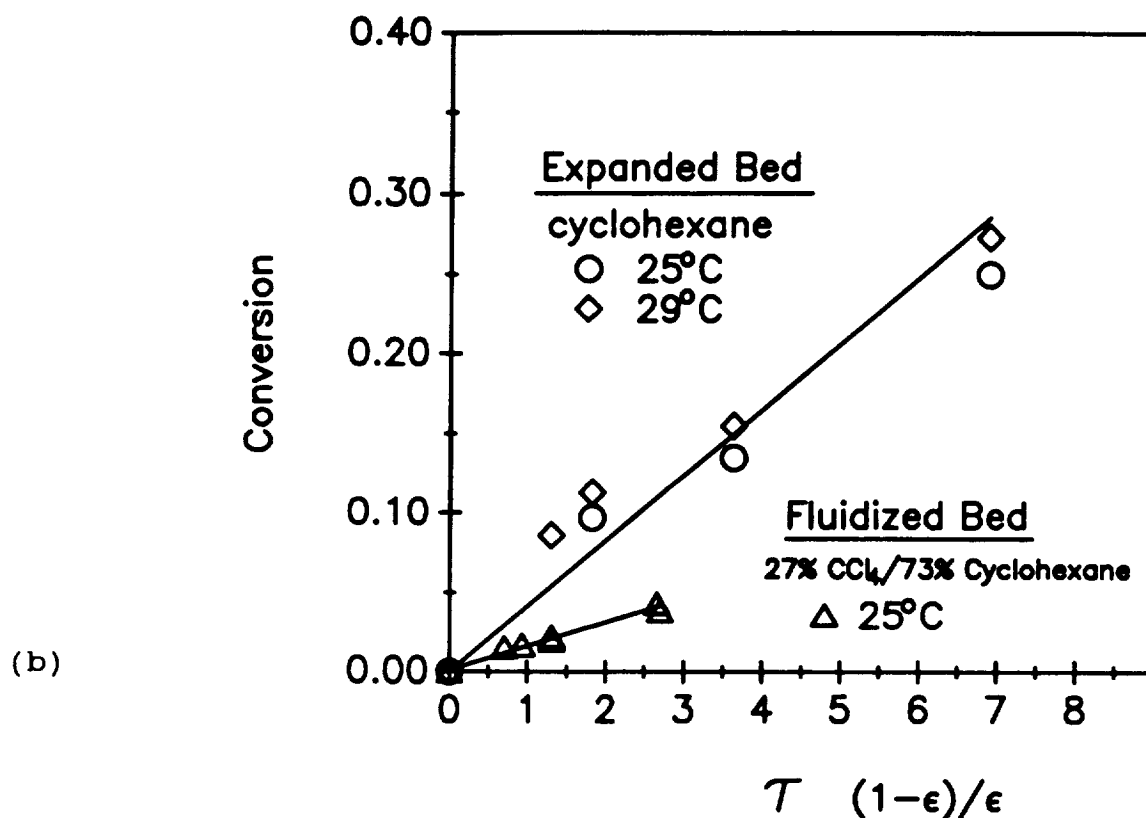
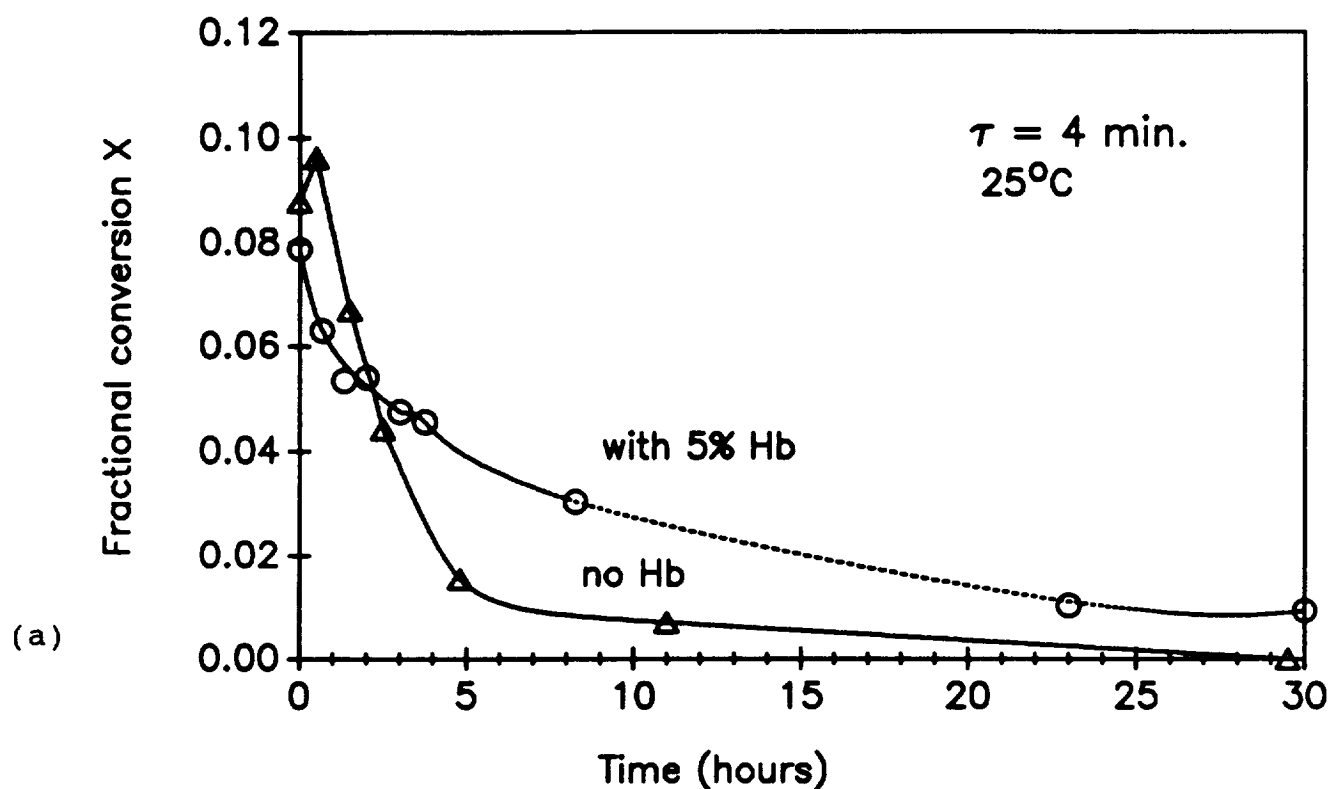


Figure 3-5. Micelle bioreactor kinetics. a) The effect of hemoglobin on the stability of YADH in microcapsules. b) The effect of residence time on conversion in expanded and fluidized bed reactors.

immobilized form was higher than in the free suspended form. The enzyme immobilized on Celite 560 displayed the highest activity. The enzyme was immobilized by adding a concentrated and buffered lipase solution to the dry support in a quantity enough to wet the support. The wet lipase impregnated support was then lyophilized to give approximately a monolayer of enzyme on the particle surface. The pH of the enzyme solution used for immobilization was optimized for maximum activity. Initial optimization of water content has also been performed.

The resulting enzyme activity in catalyzing interesterification of tripalmitin with stearic acid in petroleum ether which we have measured was of the order of 10,000-20,000 units/mg protein (a very encouraging starting point). Depending on the reaction conditions, we could obtain a rather high degree of stearic acid incorporation into tripalmitin: up to 50% of the total glyceride fatty acid content. The actual interesterification of palm oil has been attempted too. The interesterification reaction rate observed was comparable to that seen with tripalmitin, and the degree of stearic acid incorporation achieved was also similar. The tripalmitin system's GC analysis is simpler and more informative as compared to the palm oil. As their behavior in this reaction is similar, we will devote most of our time initially to the tripalmitin system and shift to palm oil only towards the end of the project.

With these initial parameters optimized we are ready to start our systematic study of the factors that will eventually determine the overall enzymatic reactor efficiency. These factors include water content, support properties and immobilization configuration, short term and long term stability and the molecular basis for the "pH memory" of enzymes in organic solvents.

C. Process Design and Analysis

- (1) Bioprocess Synthesis, Integration, and Analysis (J. D. Ingham and N.K. Rohatgi, Jet Propulsion Laboratory). The purpose of this task is to develop computer modeling and process simulation methods for rapid, realistic assessments of new bioprocess concepts, and to derive or synthesize a series of candidate bioprocesses and systematically conduct relevant energy-economic analyses and comparisons for added-value commodity chemicals to determine the potential for process technology transfer and commercial

development. These bioprocesses will also be modified to include significant research advances to be evaluated.

Kinetics models have been developed to allow calculations for batch, continuous stirred tank (cstr) and tubular plug flow (pfr) bioreactors. The latter would result in the highest rate of production (productivity), but in tubular bioreactors there is considerable axial mixing, primarily because of the presence of an evolving vapor phase, with the result that actual productivity will be greater than for a cstr and less than for an ideal pfr where plug flow is assumed. For these models specific kinetic parameters that are usually determined experimentally in batch processes are needed. The usual values determined are the maximum specific cell growth rate, inhibition and saturation constants for cell growth, and yield coefficients. The equations based on such cell growth parameters are not consistent with corresponding equations based on product parameters. In reported work [cf., e.g., Ghose and Tyagi, *Biotechnol. and Bioeng.*, 21, 1401-1420 (1979)] experimentally determined yield coefficients are constant; however, they are only constant within experimental error at concentrations up to about 50 g/L. Yield coefficients cannot be constant as ethanol concentration changes because yields of product relative to cells depend on the ratio of the rate of product formation to cell formation and this ratio increases as reaction proceeds. The rate ratio is infinite at ethanol concentrations >87 g/L, when cell production ceases. If ethanol production stops at a higher product concentration (ethanol: 114 g/L) than cell growth (inhibiting concentration of ethanol: 87 g/L), then it is obvious that at concentrations >87 g/L the yield coefficient for cells (g cells/g substrate) is zero. In the current model it is assumed that Y_{ps} (as g ethanol/g substrate) increases as reaction proceeds since more substrate is being converted to ethanol and less is being converted to cells. When cell concentration is essentially constant as in an ideal pfr with rigidly immobilized cells (e.g., as in a uniformly packed bed, but not necessarily for a fluidized bed), the yield coefficient may correspond to only product formation, but it is not assumed to be at the maximum (0.511) because although cell concentration may be nearly constant, at some concentrations cells are being formed, and under other conditions they may lose viability. It should also be pointed out that if the maximum specific rate of ethanol production is 1.4 and the maximum specific rate of cell growth is 0.4,

then at high substrate concentration and low product concentration, the relative rates are near 1.4/0.4, or 3.5. This means that under these conditions the yield coefficient as g alcohol/g cells is 3.5, yet the yield coefficient determined experimentally by Ghose and Tyagi was 5.2 for these values of maximum specific rates. It was expected that calculations might show that the value of 3.5 approaches 5.2 as reaction proceeds, because of differences in the saturation constants, but that is not the case. The ratio of maximum rates should be closer to the yield coefficient. In the current model calculations, the observed maximum relative rates and initial yield coefficients have been used, but equations have been included to reflect changes in yield coefficients as reaction proceeds.

When product parameters are not available, the cell growth equation is not defined at concentrations greater than the limit of 87 g/L, and in some cases it has been concluded that about 90 g/L is the maximum ethanol concentration obtainable when yeast is used. This conclusion is not valid; many (if not most) batch ethanol fermentations are operated to concentrations of 100 g/L or higher. If product parameters are not available, it can be assumed that the inhibition constant changes for ethanol production and is about 30% higher than for cell growth. This is based on the referenced work, and seems reasonable for yeast because it has been generally observed that product formation continues at constant cell concentration. Work is in progress to attempt to estimate kinetic behavior, based only on cell growth parameters and minimal experimental data, for product concentrations that are higher than the limiting concentration for cell growth.

Further task accomplishments include:

- (a) For the proposed acetaldehyde process, the base-case process mass balance has been calculated, the energy assessment has been started and potential process improvements to minimize the ethanol recycle stream are being evaluated.
- (b) For ethyl acetate, energy assessments for three processing approaches for ethanol separation (CO_2 stripping, vacuum fermentation, and vacuum fermentation followed by fractionation) have been evaluated; vacuum fermentation requires the least energy (1600-2000 Btu/lb of ethyl acetate produced). Detailed design of the catalytic reactor has been completed.

- (c) Progress has been made in programming existing codes from Basic to Fortran (to be more compatible with commercial simulators) and initiation of the preparation of instructional documentation for program utilization.
 - (d) A new technology report, "Synthesis of Ethyl Acetate from Fermentation Ethanol" by N.K. Rohatgi and J.D. Ingham has been prepared for preliminary evaluation of potential patentability.
 - (e) Procurements for two contracts, one with the U. of Ma. (PI: J. Douglas) and the other with SERI (PI: G. Hinman) had been initiated for bioprocess synthesis and assessments, respectively. Professor Douglas is no longer available, and the Massachusetts procurement has been cancelled. Work on process synthesis modeling will be started at JPL.
- (2) Economic Evaluations of Potential Bioprocesses (R.M. Busche, Bio-EGN Associates). The objective was to determine the economics of several bioprocess concepts to attempt to establish if they could lead to profitable commercial processes. (The National Corn Growers Association was a cosponsor for this work.) Conceptual processes are: (a) n-butanol, where the products are extracted from the broth; (b) recombinant aerobes for production of specialty chemicals; (c) production of ethanol using Zymomonas mobilis or yeast; and (d) extractive fermentation for production of acetic acid.
- (a) The purpose is to continuously remove product as formed to minimize rate inhibition by the product. Although the results indicate that n-butanol could be produced profitably from corn syrup at a selling price as low as \$0.43/lb (compared to a current price of about \$0.38/lb for production from propylene), there are many problems associated with production of this alcohol from sugar feedstocks. In addition to problems addressed in this work (e.g., a maximum butanol concentration of ~1 wt%, utilization of large quantities of solvent for recovery, and requirements for an extensive distillation complex to recover n-butanol and side products), two additional obstacles are: (i) there is no known solvent that has the necessary properties for economical extraction of n-butanol, and numerous previous attempts to discover or develop such a

solvent have not been successful, and (ii) the microorganism used, Clostridium acetobutylicum, does not produce n-butanol as consistently as required for continuous processes. In this work, the solvent is a hypothetical solvent with low water solubility, and other desirable properties such as a relatively high volatility and distribution coefficient. The rates of production of acids (such as n-butyric acid, which is later biologically converted to product alcohol) and production of n-butanol are dependent on the stage in the life cycle, or age, of each cell. As a result, laboratory-scale production of n-butanol by continuous fermentation bioprocesses has not been clearly demonstrated. In all cases the actual product concentration in the broth or extractant has been very low, or the time for continuous operation has been limited to about two days.

Although further research should minimize existing obstacles to direct production of n-butanol by a biocatalyzed process, a potential alternative is production from fermentation ethanol as the primary feedstock, for further conversion to added-value n-butanol, where the technical problems and economic constraints would seem to be less formidable.

- (b) This type of process will have no significant impact on national energy consumption because none of the products assessed are produced on a large scale. Use of recombinant aerobes to increase oxygen transfer and cell growth rates would significantly decrease absolute plant investment costs for high value products. The relative costs tend to be masked by relatively high labor and other costs, and higher planned returns, since only fermenter costs are affected. In this assessment it was concluded that investment reductions may be accomplished by increasing product concentration, cell density and specific productivity, and by converting to continuous operation. Actually, this conclusion applies to essentially all biocatalyzed production processes, as shown by many others.
- (c) A similar assessment by Chem Systems Inc. made in the early 80's indicated only minor cost reductions (2-3%) by using the bacterium instead of yeast. In this assessment it was assumed that higher concentrations of ethanol can be obtained

(110 g/L vs. 90 g/L for yeast) and the cost advantage for Z. mobilis is then about 5-10%. In fact it has never been established that concentrations higher than 80 g/L can be obtained in any efficient continuous ethanol bioprocess, or that the bacterium is any more alcohol tolerant than yeast, and, therefore the potential cost differential is relatively small. Energy consumption is the same if product concentration is the same, as we have shown in earlier work.

- (d) In this concept, acetic acid is recovered by solvent extraction from the broth. The purpose is to continuously remove product as formed to minimize rate inhibition by product and to recover acetic acid by a more efficient method than distillation. Distillation is not economically practical because all the water (>10 lb/lb of acid) would have to be distilled from the product, which has a higher boiling point than water. The results of this assessment show that much more efficient microorganisms would have to be developed before this conceptual bioprocess type would be economically competitive with the current industrial process (carbonylation of methanol). As is the case for n-butanol, a better approach may be to concentrate efforts on improving ethanol bioprocesses, since ethanol can be readily converted to acetic acid. This contract has been completed on schedule as planned.

SECTION IV

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